Potential targets for next generation antimicrobial glycoconjugate vaccines

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One sentence summary: This review is intended to analyze structural and functional differences of surface-exposed polysaccharides from emerging pathogenic bacteria and, combined with epidemiological, medical need and technological considerations, identify potential targets for glycoconjugate vaccines in the near future. How advances in the field of glycoconjugate vaccine production (conjugation of natural polysaccharides, chemo-enzymatic approaches, glycoengineering) can support the development of efficacious novel well-defined vaccines is also discussed.

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ABSTRACT

Cell surface carbohydrates have been proven optimal targets for vaccine development. Conjugation of polysaccharides to a carrier protein triggers a T-cell-dependent immune response to the glycan moiety. Licensed glycoconjugate vaccines are produced by chemical conjugation of capsular polysaccharides to prevent meningitis caused by meningococcus, pneumococcus and *Haemophilus influenzae* type b. However, other classes of carbohydrates (O-antigens, exopolysaccharides, wall/teichoic acids) represent attractive targets for developing vaccines. Recent analysis from WHO/CHO underpins alarming concern toward antibiotic-resistant bacteria, such as the so called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) and additional pathogens such as *Clostridium difficile* and Group A *Streptococcus*. Fungal infections are also becoming increasingly invasive for immunocompromised patients or hospitalized individuals. Other emergencies could derive from bacteria which spread during environmental calamities (*Vibrio cholerae*) or with potential as bioterrorism weapons (*Burkholderia pseudomallei* and *mallei*, *Francisella tularensis*). Vaccination could aid reducing the use of broad-spectrum antibiotics and provide protection by herd immunity also to individuals who are not vaccinated.

This review analyzes structural and functional differences of the polysaccharides exposed on the surface of emerging pathogenic bacteria, combined with medical need and technological feasibility of corresponding glycoconjugate vaccines.

Keywords: carbohydrates; glycoconjugates; vaccines; glycoengineering; antimicrobial resistance

INTRODUCTION

Surface carbohydrates, particularly capsular polysaccharides (CPS), have been proven optimal targets for bacterial vaccines development. Polysaccharide-based vaccines against meningococcus, pneumococcus and *Haemophilus influenzae* type b were licensed between the 1970s and the early 1980s. Due to their T-cell independent character, they are efficacious in adults, but fail to elicit adequate protection in high-risk groups, such as infants and children under 2 years of age (Peltola *et al.* 1977a,b).

This limitation of polysaccharide vaccines can be overcome by conjugation to a carrier protein, which triggers a T-cell-dependent immune response to the carbohydrate moiety (Costantino, Rappuoli and Berti 2011) and assures efficacious vaccination of children and elderly.

Glycoconjugate vaccines have been used to control a variety of bacterial infections in recent years, and more vaccines are

either under development at preclinical level or in clinical trials (Costantino, Rappuoli and Berti 2011).

The glycoconjugate vaccines licensed so far are obtained from CPS or derived fragments (Table 1). However, pathogenic bacteria also display other classes of carbohydrates that might represent good candidates for vaccine development, especially when the pathogen does not produce a capsule (e.g. most of the *Shigella* species or *Vibrio cholerae*) or the capsule mimics self-structures (e.g. α-(2→8) polysialic acid capsule of *Neisseria meningitidis* serogroup B, and the polyhyaluronic acid capsule of Group A *Streptococcus*) or the pathogen has a high number of strains with different CPS, making vaccine formulation development very complicated.

In these cases, other glycans, such as the O-antigen portion of lipopolysaccharide (LPS) molecules in Gram-negative or cell wall-associated glycans in Gram-positive bacteria, can be sufficiently accessible to the immune system to be taken into consideration as vaccine candidates. Notable examples are *V. cholerae* (Sayeed *et al.* 2015), *Shigella* species (Mani, Wierzba and Walker 2016) and *Escherichia coli* (Huttner *et al.* 2017).

The emerging of antimicrobial resistance (AMR) for some pathogens including, among others, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Clostridium difficile*, which are currently not treated by vaccination, is rendering the identification of future candidates more urgent (Garcia-Quintanilla *et al.* 2016). In fact, vaccination could aid reducing the use of broad spectrum antibiotics and provide protection (herd immunity) also for individuals who are not vaccinated (Lipsitch and Siber 2016).

This review is intended to analyze structural and functional differences of surface-exposed polysaccharides from emerging pathogenic bacteria and, combined with epidemiological, medical need and technological considerations, identify potential targets for glycoconjugate vaccines in the near future. How advances in the field of glycoconjugate vaccine production (conjugation of natural polysaccharides, chemo-enzymatic approaches, glycoengineering) can support the development of efficacious novel well-defined vaccines is also discussed.

THE SURFACE CARBOHYDRATE STRUCTURES OF BACTERIA AND FUNGI

The bacterial cell envelope is surrounded by a dense layer of fibrous polysaccharides and glycoproteins, named *glycocalyx*. This structure helps bacteria to survive in unpredictable and often hostile environment, while it allows the selective passage of nutrients from the outside and waste products from the inside.

Generally, the capsule is the outermost surface polysaccharide of both Gram-negative and Gram-positive bacteria (Hendrickx *et al.* 2011; Brown *et al.* 2015; Filloux and Whitfield 2016). A given bacterial species might have strains with structurally different CPS resulting in different serogroups or serotypes. Immediately below the capsule, Gram-negative bacteria are characterized by an outer membrane (OM) from which anchored LPS, CPS and membrane proteins protrude. A thin peptidoglycan cell wall is sandwiched between OM and the inner cytoplasmic cell membrane. In contrast, Gram-positive bacteria lack an OM, and are surrounded by a much thicker layer of peptidoglycans compared to Gram-negatives (Fig. 1). Carbohydrates, like β-glucans, mannans, and others, are predominant components of the surface of fungal species (Gow, Latge and Munro 2017). Furthermore, glycosylphosphatidylinositol molecules, which are present on the surface of virtually all

eukaryotic cells serving as surface protein anchors, occur at relatively high levels and with specific structures in parasitic protozoa, such as *Plasmodium falciparum* (Gowda, Gupta and Davidson 1997). The use of parasite carbohydrates as potential vaccine antigens has been recently reviewed and will not be in the scope of the present work (Jaurigue and Seeberger 2017).

A general description of the different glycans present in bacteria and fungi is given below, and structures of glycans with potential of being used to extend the coverage of licensed glycoconjugate vaccines or for the development of future vaccines are given in Table 2.

Polysaccharide capsule

CPS are typically made of negatively charged and highly hydrophilic long-chain polysaccharides, firmly anchored to the cell membrane.

CPS are well-established virulence factors, and they can interfere with innate immunity preventing the activation of the alternate complement pathway. Their hydrophilic character protects microorganisms from desiccation, thus facilitating host-to-host transmission, and their chemical structure sometimes mimics molecules produced by human cells so that the pathogen is not recognized as foreign by the immune system.

A paradigm of the remarkable structural diversity in CPS is embodied by almost 80 capsular serotypes in *E. coli*, more than 90 in *Streptococcus pneumoniae* and about 70 capsular serotypes in *K. pneumoniae* (Willis and Whitfield 2013). The most studied system for the biosynthesis of CPS is *E. coli* (Whitfield 2006). Its CPS are classified into four groups, numbered from 1 to 4 on the basis of genetic and biosynthetic criteria. In terms of biosynthesis three main pathways have been identified (Fig. 2): Wzx/Wzy, ATP-binding cassette (ABC) transporter and synthase dependent (Cuthbertson, Kos and Whitfield 2010; Willis and Whitfield 2013). The Wzx/Wzy- and ABC transporter-dependent pathways share some similarities: the polysaccharide is built on a lipid acceptor, usually undecaprenol diphosphate (UndPP), starting from activated building blocks, that are typically cytosolic sugar nucleotides (Fig. 2A). Glycosyltransferase reactions transfer sugars to UndPP, at the cytoplasmic face of the membrane. The final lipid-linked polysaccharide is located outside the cytoplasmic membrane. In the Wzx/Wzy-dependent pathway, the individual repeating units are first assembled and then exported across the membrane by a flippase Wzx protein. The exported UndPP-linked glycans are finally polymerized by a Wzy polymerase, which extends the growing chain one repeat unit at a time at the periplasmic face of the cytoplasmic membrane. A polysaccharide copolymerase controls the polymerization process. Groups 1 and 4 CPS are synthesized according to Wzy-dependent processes. These types of CPS are found in isolates causing intestinal infections, including enteropathogenic, enterotoxigenic and enterohemorrhagic *E. coli,* and other relevant pathogens, comprising *St. pneumoniae* and *S. aureus* (Yother 2011).

In the ABC transport-dependent pathway, the polysaccharide is completed at the cytoplasmic face of the inner membrane (IM), and then exported by the ABC transporter (Fig. 2B). Group 2 and 3 capsules, generally found in isolates causing extra intestinal infections, are both assembled via this pathway. Their structural features vary extensively and seem reminiscent of *N. meningitidis* and *H. influenzae* CPS (Whitfield 2006).

In the synthase-dependent pathway (Fig. 2C), which is the less known of the three mechanisms, a polymerizing glycosyltransferase (the synthase) assembles the polysaccharide at the cytoplasmic face of the IM and is also believed to be involved in its export across the IM (Willis and Whitfield 2013). Serotypes

Table 1. Examples of glycoconjugate vaccines in the market or in development.

Type of glycan	Organism (WHO/CDC category of AMR top T high H medium M)	Manufacturer (licensed :L) (clinical:C) (discovery: D)	Saccharide	Approach[a]	Carrier	Ref
Capsular polysaccharide	Hib (M)	GSK (L)	PS	SS	TT	CDC (2016)
		Sanofi (L)	PS	SS	TT	Zou and Jennings (2009)
		GSK (L)	Oligo	SS	CRM197	Costantino, Rappuoli and Berti (2011)
		Merck (L)	Size reduced PS	SS	OMPC	Marburg et al. (1986)
		Pfizer(L)	Oligo	SS	CRM197	Anderson et al. (1986)
		SIIL (L)	PS	SS	TT	Sharma et al. (2012)
		CIGB (L)	Oligo	ST	TT	Verez-Bencomo et al. (2004)
		Hilleman Lab (D)	Size reduced PS	SS	TT	Laferriere et al. (2011; Rana et al. 2015; Schneerson et al. 1980)
	Hia	Bionet-Asia NRC Canada (D)	Size reduced PS	SS	CRM197 and Protein D	Cox et al. (2017)
	Meningococcus	GSK (L)	Oligo MenC	SS	CRM197	Costantino, Rappuoli and Berti (2011)
		Pfizer (Nuron) (L)	MenC size reduced PS	SS	CRM197	Ravenscroft, Wheeler and Jones (2010)
		Baxter (L)	MenC PS De-OAc Size reduced	SS	TT	Ravenscroft, Wheeler and Jones (2010)
		Hilleman Lab (D)	MenX	ST	TT	Harale et al. (2015)
		SIIL (L)	MenA Size reduced PS	SS	TT	Ravenscroft, Wheeler and Jones (2010)
		GSK (D)	MenX Ps size reduced	SS	CRM197	Fiebig et al. (2017); Micoli et al. (2013)
		GSK (L)	MenACWY Oligos	SS	CRM197	Broker et al. (2009)
		Pfizer (L) formerly GSK	MenACWY size reduced PS	SS	TT	Broker, Berti and Costantino (2016)
		Sanofi (L)	MenACWY size reduced PS	SS	DT	Ravenscroft, Wheeler and Jones (2010)
		Sanofi (C)	MenACWY	SS	TT	McVernon et al. (2012)
		SIIL (C)	MenACWYX PS	SS	TT, CRM197	LaForce (2017)
	Pneumococcus (M/H)	Pfizer (L)	4, 6B, 9V, 14, 18C, 19F, 23F, PS except 18C size reduced	SS	CRM197	Ravenscroft et al. (2015)
		Pfizer (L)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F PS except 18C size reduced	SS	CRM197	Ravenscroft et al. (2015)
		GSK (L)	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F PS except 23F size reduced	SS	Protein D, TT(18C), DT (19F)	Dhillon and Pace (2017)
		Limmatech Biologics (D)	Multivalent	B	rEPA	Ravenscroft et al. (2017)
		Merck (C)	15 valent	SS	CRM197	McFetridge et al. (2015)
		CIGB (C)	1, 5, 6B, 14, 18C, 19F, 23F	NA	TT	Linares-Perez et al. (2017)
	GBS (na/M)	GSK (C)	Ia, Ib, III PS	SS	CRM197	Madhi et al. (2013)
		GSK (D)	Ia, Ib, II, III, V PS	SS	CRM197	Kobayashi et al. (2016)

Table 1. – Continued.

Type of glycan (high H medium M)	Organism (WHO/CDC category of AMR top T high H medium M)	Manufacturer (licensed :L)(clinical:C)(discovery: D)	Saccharide	Approach[a]	Carrier	Ref
		Various (D or C); Pfizer (C)	Ia, Ib, II, III, IV, V, VI, VII and VIII Ps; Multivalent	SS; Platform developed for pneumo conjugates	TT and CRM197; CRM197	Heath (2016); Kobayashi et al. (2016)
	Staphylococcus aureus (H)	GSK (C)	Type 5 and 8 PS	SS	TT	Levy et al. (2015)
		Pfizer (C)	Type 5 and 8 PS	SS	CRM197	Nissen et al. (2015); Frenck et al. (2017)
		GlycoVaxyn (now Limmatech Biologics) (D)	Type 5 and 8 PS	B	rEPA	Wacker et al. (2014)
	Salmonella Typhi (H/M)	NIH (C), GVGH/Biological E (C), Biomed (L), Barath Biotech (L)	Vi PS and Fragments	SS	CRM197, TT, DT, rEPA	MacLennan, Martin and Micoli (2014)
	Burkholderia pseudomallei	DSTL (D)	Oligo	ST	TetHc	Scott et al. (2016)
	Klebsiella pneumoniae (T/T)	Max Plank Institute (D)	CPS repeating unit	ST	CRM197	Seeberger et al. (2017)
	Shigella (M/H)	Limmatech Biologics (C)	Sh. dysenteriae type 1 PS; Sh. flexneri 2a PS	B	rEPA	Hatz et al. (2015); Riddle et al. (2016)
		NICHHD (C)	S. sonnei and Sh. flexneri 2a PS	SS	rEPA	Ashkenazi et al. (1999)
		Institute Pasteur (C)	Sh. flexneri 2a oligo	ST	TT	van der Put et al. (2016)
O-Antigen	Escherichia coli	Limmatech Biologics/J&J (C)	O1, O2, O6, O25 Expec	B	rEPA	van den Dobbelsteen et al. (2016)
	Salmonella Paratyphi A and non-typhoidal Salmonella (H/M)	NVGH (D), NIH (C), IVI (D)	O2 S. Paratyphi A, O9 S. Enteritidis, O4,5 S. Typhimurium	SS	TT, CRM197, DT	MacLennan, Martin and Micoli (2014)
	Pseudomonas aeruginosa (T/H)	SSVI/WRAIR (C) program stopped	O1,2,3,4,5,6,11,12	SS	EPA	Cryz et al. (1987, 1989); Lang et al. (2004); Schaad et al. (1991)
	Klebsiella pneumoniae (T/T)	University Maryland (D)	O1, O2a, O2a,c, O3, O4, O5, O7, O8, O12	SS	PA flagellin	Simon, Cross and Tennant (2016)
	Vibrio cholerae	NIH, Institut Pasteur (D)	O1 (Inaba and Ogawa), O139	SS; ST	BSA, rEPA, TThc	Gupta et al. (1998); Boutonnier et al. (2001); Chernyak et al. (2002); Wade et al. (2006); Rollenhagen et al. (2009); Alam et al. (2014); Sayeed et al. (2015); Soliman and Kovac (2016)
	Francisella tularensis; Burkholderia pseudomallei	CCRC-NRCC and DSTL (D); Academic (D)	O-Ag; OPSII	ST; B; B; ST	KLH; rEPA; AcrA;	Boltje et al. (2012); Cuccui et al. (2013); Garcia-Quintanilla et al. (2014); Kenfack et al. (2017)
	Moraxella catarrhalis	NRC Canada (D)	Truncated LPS	SS	CRM197	Cox et al. (2011)
		NDCD/NIH (D)	Detox LPS serotype A, B and C	SS	TT, NTHi HMP, UspA, CD, CRM197	Gu et al. (1998); Hu et al. (2004); Yu and Gu (2005, 2007)

Table 1. – Continued.

Type of glycan	Organism (WHO/CDC category of AMR top T high H medium M)	Manufacturer (licensed :L) (clinical:C) (discovery: D)	Saccharide	Approach[a]	Carrier	Ref
PNAG Teichoic acids	Enterococcus faecalis (H/H)	UML/Leiden University (D)	LTA	ST	BSA	Laverde et al. (2014)
	Acinetobacter baumannii (T/H) and other pathogens	Harvard Medical School, Alopexx (D)	β-(1→6)-oligo glucosamine	ST	TT	Cywes-Bentley et al. (2013); Gening et al. (2009)
ExoPS	Pseudomonas aeruginosa (T/H)	Harvard Medical School (C and D)	Polymannuronic acid; alginate	ST	ExoA, Flagellin; TT, KLH, OMV, synthetic peptides	Campodonico et al. (2011); Doring and Pier (2008); Farjah et al. (2015); Farjah et al. (2014); Kashef et al. (2006); Theilacker et al. (2003)
Cell Wall PS	Clostridium difficile	Guelph University, Max Planck Institute (D)	PS-I	ST	CRM197	Broecker et al. (2016a); Martin et al. (2013b)
		GSK, Guelph University, Max Planck Institute (D)	PS-II	ST; SS	CRM197, Clostridiumdifficile rToxins,	Adamo et al. (2012); Bertolo et al. (2012); Romano et al. (2014)
		Max Planck Institute (D)	PS-III	ST	CRM197	Broecker et al. (2016b); Cox et al. (2013); Martin et al. (2013a)
	Group A Streptococcus (GAS) (M/M)	GSK (D)	GAC fragments	ST	CRM197	Kabanova et al. (2010)
		Rockefeller University	PS	ST	TT	Sabharwal et al. (2006)
		Various Academic Institutions (D)	GlcNAc deficient PS	ST	Sp0435	van Sorge et al. (2014)
Fungal glycans	Aspergillus fumigatus	Zelinsky Inst. Org. Chem./Institute Pasteur (D)	α-(1→3)-glucans	ST	BSA	Komarova et al. (2015)
	Candida albicans (na/M)	GSK, CCRC (D)	β-(1→3)/β-(1→6)-glucans	SS; ST	CRM197	Adamo et al. (2011, 2014); Bromuro et al. (2010); Liao et al. (2015, 2016); Torosantucci et al. (2005)
		Alberta University/ Theracarb/Novadigm (D)	β-(1→2)-mannotriose	ST	TT, Candida peptides	Johnson and Bundle (2013); Xin et al. (2008)
	Cryptococcus neoformans	Dublin University/J. Hopkins Bloomberg SPH (D)	GXM PS and oligosaccharides	SS; ST	HSA	Casadevall et al. (1992); Devi (1996); Guazzelli, McCabe and Oscarson (2016); Nakouzi et al. (2009)
Mycobacterial glycans	Mycobacterium tuberculosis (H)	Uppsala University/Eurocine AB (D)	AM	SS	Ag85B, TT	Hamasur et al. (2003); Kallenius, Pawlowski and Hamasur (2008)

[a]Semisynthetic conjugates from natural carbohydrates: SS; Conjugates synthetic carbohydrates: ST; Bioconjugates: B; not available: na.
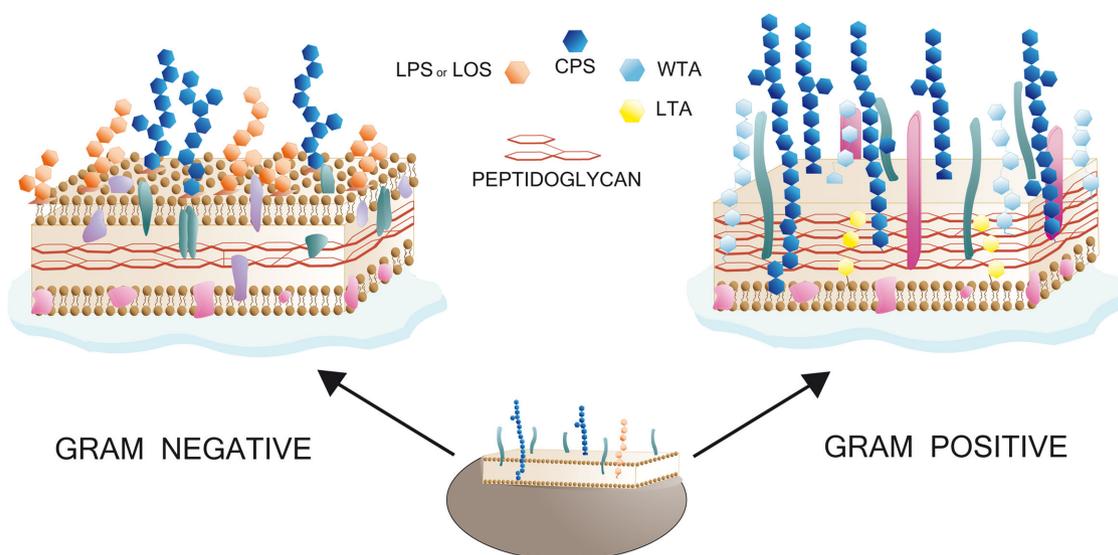
Figure 1. Structures of the cell walls of Gram-negative and Gram-positive bacteria. Both classes of bacteria can produce a capsule (CPS). Gram-negative bacteria express lipopolysaccharide (LPS) or lipooligosaccharide (LOS). Unlike Gram-negative bacteria which possess an outer membrane with an outmost layer rich of phospholipids and LPS molecules, Gram-positive bacteria lack of the outer membrane and possess lipoteichoic acids (LTA) and the more exposed wall teichoic acids (WTA).

3 and 37 of *St. pneumoniae* are known to follow the synthase-dependent pathway for their CPS biosynthesis (Yother 2011).

Unlike the CPS biosynthesis, structure and biochemical pathway for the anchor to the cell membrane is less known. In *E. coli*, *N. meningitidis, H. influenzae* and other Gram-negative pathogens, this anchor is made of (lyso)phoshatidylglycerol moiety to which CPS is attached via an oligosaccharide of five to nine β-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues (Fig. 3A) (Willis *et al.* 2013). The CPS of *Salmonella* Typhi (Vi antigen) has a unique lipid terminus composed of a reducing terminal HexNAc residue modified with two β-hydroxy fatty acids that resembles one half of lipid A structure (Liston, Ovchinnikova and Whitfield 2016). Some bacteria, in addition to the LPS molecules carrying the serological O-antigen, produce a CPS linked to a lipid A core, and therefore termed K_{LPS} or K antigen (Whitfield 2006).

In most of capsule-forming Gram-positive bacteria, the majority of the polymers is covalently linked to the peptidoglycans or to membrane components, although some may be released from the cell (Yother 2011). There are exceptions, such as *St. pneumoniae* type 3, where the CPS is bound to the membrane through a phosphatidylglycerol anchor (Cartee, Forsee and Yother 2005).

Glycans associated to Gram-negative bacteria OM

The OM is a distinguishing feature of Gram-negative bacteria. Unlike most biological membranes, the OM is an asymmetrical lipid bilayer. Typically, the inner leaflet is composed predominantly of phospholipids and the outer leaflet of LPS (Raetz and Whitfield 2002; Filloux and Whitfield 2016).

The human innate immune system is sensitized to LPS which is generally an indicator of infection. LPS is responsible for the endotoxic shock associated with the septicemia caused by Gram-negative organisms (Raetz and Whitfield 2002). LPS is made of three components (Fig. 3B): lipid A, core-oligosaccharide and O-polysaccharide (O-PS) or O-antigen (O-Ag). The OM generally contains a complex mixture of LPS molecules, including molecules with only the lipid A and core-oligosaccharide (rough LPS), as well as molecules capped with O-PS (smooth LPS) (Knirel *et al.* 2001).

The typical lipid A structure consists of a glucosamine disaccharide, substituted with fatty acids (Raetz and Whitfield 2002). The acyl chains are largely saturated and facilitate tight packing of OM, playing a critical role in the barrier function of the OM. The core-oligosaccharide is divided into two regions: the inner core consisting of Kdo and L-glycero-D-manno-heptose residues that is highly conserved, and the outer core, which displays limited structural diversity and consists mainly of hexose sugars. The O-Ag domain is made up of repeating units of one or more sugar residues and exhibits remarkable structural diversity. Variations in its composition are often the basis for serotyping classification by serological methods. Although not essential for growth in laboratory culture, O-Ag helps the bacterium to resist certain antimicrobial molecules, the complement system, and environmental stresses in its natural environment (Raetz and Whitfield 2002; Greenfield and Whitfield 2012).

Some Gram-negative bacteria, such as *Neisseria* spp., *Haemophilus* spp. and *Bordetella pertussis*, and in general mucosal pathogens are unable to synthesize O-Ag and produce instead a LPS form called lipooligosaccharide (LOS) that contains the inner core from which one or more mono- or oligosaccharide branches (which determine serological specificity) extend. *Pseudomonas aeruginosa* can produce a rough LPS, once colonization has been established (Knirel *et al.* 2001).

Biosynthesis of the lipid A is a highly conserved process among Gram-negative species, which occurs partly in the cytoplasm, and partly at the inner leaflet of the IM (Greenfield and Whitfield 2012). The O-Ag is assembled following the same pathways than the CPS, except that the ABC transporter-dependent mechanism of O-Ag biosynthesis seems the most widespread (Fig. 2). The completed O-Ag is transferred from the UndPP linked intermediate and ligated to the lipid A-core in the periplasmic face of the IM. Very seldom the synthase-dependent mechanism is involved.

Glycans associated to Gram-positive bacterial cell wall

Peptidoglycan
Peptidoglycan (PG) is made up of repeating units of the disaccharide N-acetyl glucosamine-N-acetyl muramic acid, which are

Table 2. Structures of some surface microbial carbohydrates tested as vaccine antigens in preclinical studies or in early clinical phase.

Pathogen	Glycan type	Structure	Ref.
Bacteria			
A. baumannii	K1 CPS	→3)-β-D-QuipNAc4NR-(1→4)-α-D-GlcpNAc6OAc-(1→4)-α-D-GalpNAcA-(1→ R = OH, CH₃CHOHCH₂CO	(Russo et al., 2013)
A. baumannii	PNAG	→6)-β-D-GlcpNAc-(1→	(Bentancor et al., 2012)
B. pseudomallei	CPS (OPSI)	→3)-β-D-6dManHepp2OAc-(1→	(Knirel et al., 1992; Perry et al., 1995)
B. pseudomallei	O-Ag (OPSII)	→3)-β-D-Glcp-(1→3)-α-L-6dTalp2R-(1→ R = OAc or OMe (33%)	(Knirel et al., 1992; Perry et al., 1995)
C. difficile	PSI	→4)-α-L-Rhap-(1→3)-β-D-Glcp-(1→4)-α-D-Glcp-(1→2)-α-D-Glcp-(1-P→ 3 ← 1 α-L-Rhap	(Ganeshapillai et al., 2008)
C. difficile	PSII	→6)-β-D-Glcp-(1→3)-β-D-GalpNAc-(1→4)-α-D-Glcp-(1→4)-β-D-GalpNAc-(1→3)-α-D-Manp-(1-P→ 3 ← 1 β-D-Glcp	(Ganeshapillai et al., 2008)
C. difficile	LTA (PSIII)	P ← 6 →6)-α-D-GlcpNAc-(1→3)-α-D-GlcpNAc-(1→2)-GroA	(Reid et al., 2012)
E. coli O1A	OAg	→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→3)-β-L-Rhap-(1→4)-β-D-GlcpNAc-(1→ 2 ← 1 β-D-ManpNAc	(Baumann et al., 1991)
E. coli O2	OAg	→4)-β-D-GlcpNAc-(1→3)-α-L-Rhap-(1→2)-α-L-Rhap-(1→3)-β-L-Rhap-(1→ 2 ← 1 α-D-FucpNAc	(Jansson et al., 1987)
E. coli O6A	OAg	→4)-β-D-Manp-(1→3)-α-D-GlcpNAc-(1→4)-α-D-GalpNAc-(1→3)-β-D-Manp-(1→ 2 ← X X = β-D-GlcpNAc in K54 strain	(Jann et al., 1994)
E. coli O25	OAg	α-L-Rhap 1 → 3 →3)-α-L-FucpNAc-(1→3)-β-D-GlcpNAc-(1→4)-α-D-Glcp-(1→ 6 ← 1 β-D-Glcp	(Kenne et al., 1983)

Table 2. – *Continued.*

Pathogen	Glycan type	Structure	Ref.
Enterobacteriaceae	ECA	→3)-α-D-Fucp4NAc-(1→4)-β-D-ManpNAcA-(1→4)-α-D-GlcpNAc-(1→	(Männel and Mayer, 1978)
Enterecocci faecalis and *faecium*	CPS/LTA	CH_2OPO_2H-O→ →6-α-D-Glcp-1→OCH CH_2OPO_3H 2 ← 1 α-D-Glcp	(Huebner et al., 1999; Wang et al., 1999)
	DHG	→6)-β-D-Galf-(1→3)-β-D-Glcp-(1→	(Krylov et al., 2015)
F. tularensis	LOS	→4)-α-D-GalpNAcAN-(1→4)-α-D-GalpNAcAN-(1→3)-β-D-QuipNAc-(1→2)-β-D-Quip4NFm-(1→	(Vinogradov et al., 1991)
Group A *Streptococcus*	GAC	→α-L-Rhap-(1→3)-α-L-Rhap-(1→3)→ 3 ← 1 β-D-GlcpNAc	(Huang and Krishna, 1986)
H. influenzae type a	CPS	→4)-β-D-Glcp-(1→4)-D-Rib-ol-5-OPO$_2$H-O→	(Byrd et al., 1987)
K. pneumoniae type K1	K1 CPS	→4)-β-D-GlcpA-(1→4)-α-L-Fucp-(1→3)-β-D-Glcp-(1→ 2 3 X CH_3 COOH	(Erbing et al., 1976)
K. pneumoniae type K2	K2 CPS	→3)-β-D-Glcp-(1→4)-β-D-Manp-(1→4)-α-D-Glcp-(1→ 3 ← 1 α-D-GlcpA	(Corsaro et al., 2005)
K. pneumoniae serogroup O1	O-Ag	[→3)-β-D-Galp-(1→3)-α-D-Galp-(1→]$_m$-β-D-Galf-(1→[3)-α-D-Galp-(1→3)-β-D-Galf-(1→]$_n$ 3)-α-D-Galp- **D-galactan II** **D-galactan I**	(Whitfield et al., 1991)
K. pneumoniae serogroup O2a,c	O-Ag	[→5)-β-D-Galf-(1→3)-α-D-GlcpNAc-(1→]$_m$-β-D-Galf-(1→[3)-α-D-Galp-(1→3)-β-D-Galf-(1→]$_n$ 3) -α-D-Galp- **D-galactan I**	(Whitfield et al., 1992)
K. pneumoniae serogroup O3	O-Ag	α-D-Manp3OMe-(1→[2)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→3)-α-D-Manp-(1→]$_n$-3)-α-D-Manp-(1→3)-α-D-Manp-	(Vinogradov et al., 2002)
K. pneumoniae serogroup ST258	O-Ag	→3)-β-D-Galf-(1→3)-[α-D-Galp-(1→4)]-α-D- Galp-(1→ **D-galactan III**	(Szijarto et al., 2016)

Table 2. – *Continued.*

Pathogen	Glycan type	Structure	Ref.

M. catarrhalis	LOS A	α-D-GlcpNAc-(1→2)-β-D-Glcp
		  1
		  ↓
		  4
		α-D-Galp-(1→4)-β-D-Galp-(1→4)-α-D-Glcp-(1→2)-β-D-Glcp-(1→6)-α-D-Glcp-(1→5)-Kdo
		  6
		  ↑
		  1
		β-D-Glcp
			(Edebrink et al., 1994)

	LOS B	β-D-GlcpNAc-(1→4)-α-D-Galp-(1→2)-β-D-Glcp
		  1
		  ↓
		  4
		β-D-Galp-(1→4)-α-D-Galp-(1→2)-β-D-Glcp-(1→4)-α-D-Glcp-(1→5)-Kdo
		  6
		  ↑
		  1
		β-D-Glcp
			(Edebrink et al., 1996)

	LOS C	α-D-Galp-(1→2)-β-D-Glcp
		  1
		  ↓
		  4
		α-D-Galp-(1→2)-β-D-Glcp-(1→6)-α-D-Glcp-(1→5)-Kdo
		  6
		  ↑
		  1
		β-D-Glcp
			(Edebrink et al., 1995)

N. meningitidis serogroup X		→4)-D-GlcpNAc-(1P→	(Bundle et al., 1974)
P. aeruginosa O1	O-Ag	→4)-α-D-GalpNAc-(1→4)-β-D-GlcpNAc3NAcA-(1→3)-α-D-FucpNAc-(1→3)-α-D-QuipNAc -(1→	(Knirel et al., 2006)
P. aeruginosa O6	O-Ag	→3)-α-D-Rhap-(1→4)-α-D-GalpNAc3NAcA-(1→4)-α-D-GalpNFoAc-(1→3)-α-D-QuipNAc -(1→	(Knirel et al., 2006)
P. aeruginosa O11	O-Ag	→2)-α-D-Glcp-(1→3)-α-L-FucpNAc-(1→3)-β-D-FucpNAc-(1→	(Knirel et al., 2006)

Salmonella Paratyphi A	O-Ag	α-D-Galp
		  1
		  ↓
		  6
		α-D-Parp
		  1
		  ↓
		  3
		→2)-α-D-Manp-(1→4)-α-L-Rhap2/3OAc-(1→3)-α-D-Galp-(1→
			(Ravenscroft et al., 2015a)

S. aureus type 5	CPS	→4)-β-D-ManpNAc3OAcA-(1→4)-α-D-FucpNAc-(1→3)-β-D-FucpNAc-(1→	(Jones et al., 2005)
S. aureus type 8	CPS	→3)-β-D-ManpNAcA(4OAc) -(1→3)-α-D-FucpNAc-(1→3)-α-D-FucpNAc-(1→	(Jones et al., 2005)

S. aureus	WTA	→[3)-Gro-(1-P-]m-[5)-D-Rib-OH-2/3OR-(1-P→]n→
		  1
		  ↑
		  4
		β-D-GlcpNAc
		R = Ala, 50%
		
		→5)-[β-D-GlcpNAc-(1→4)]-D-Rib-OH-(1-P→
			(Saderson et al., 1962)
			
			(Vinogradov et al., 2006)

Table 2. – *Continued.*

Pathogen	Glycan type	Structure	Ref.
	LTA	$\rightarrow$[3]-Gro-(1-P-]$_m$-[-3]-Gro2OAla-(1-P]$_n\rightarrow$	(Morath et al., 2001)
S. flexneri **2a**		$\rightarrow$[3]-Gro2OR^1-(1-P-]$_n$-6)-β-D-Glc*p*-(1$\rightarrow$6)-β-D-Glc*p*-(1$\rightarrow$3)-Gro2/3OR^2 R^1 = β-D-Glc*p*NAc, Ala or H R^2 = COC$_{15}$H$_{31}$ (av)	(Perepelov et al., 2009; 2012)
		α-D-Glc*p* 1 $\downarrow$ 4 $\rightarrow$2)-α-L-Rha*p*3/4OR^1-(1$\rightarrow$2)-α-L-Rha*p*-(1$\rightarrow$3)-α-L-Rha*p*-(1$\rightarrow$3)-β-D-Glc*p*NAc6OR^2-(1$\rightarrow$ R^1 = Ac, ~60/~25% R^2 = Ac, ~60	
S. dysenteriae **type 1**		$\rightarrow$3)-α-L-Rha*p*-(1$\rightarrow$3)-α-L-Rha*p*-(1$\rightarrow$2)-α-D-Gal*p*-(1$\rightarrow$3)-α-D-Glc*p*NAc-(1$\rightarrow$	(Linnerborg et al., 1995)
V. Cholerae **O1**	OAg	α-D-Man*p*6d4NR^{1}OR^2-(1$\rightarrow$2)-[α-D-Man*p*6d4NR^1-(1$\rightarrow$2)]$_n$- R^1 = 3-deoxy-L-glycerotetronyl R^2 = CH$_3$ (Ogawa), H (Inaba)	(Hisatsune et al., 1993a)
V. Cholerae **O139**	OAg	$^-$O O $\diagdown$// P $\diagup\diagdown$ 4 6 α-D-Col*p*-(1$\rightarrow$2)-β-D-Gal*p* 1 $\downarrow$ 3 β-D-Glc*p*NAc-(1$\rightarrow$4)-α-D-Gal*p*A-(1$\rightarrow$3)-β-D-Qui*p*Ac-(1$\rightarrow$5)-Kdo 4 $\uparrow$ 1 β-D-Glc*p*	(Hisatsune et al., 1993b)

Table 2. – *Continued.*

Pathogen	Glycan type	Structure	Ref.
		prokaryota	
C albicans	Cell wall glycans	→3)-β-D-Glcp-(1→3)-[β-D-Glcp-(1→3)]m-β-D-Glcp-(1→	(Masuoka, 2004; Shibata et al., 1986)

```
→3)-β-D-Glcp-(1→3)-[β-D-Glcp-(1→3)]m-β-D-Glcp-(1→
                                     6
                                     ↑
                                     1
                       β-D-Glcp-(1→6)]n- β-D-Glcp

                    →2)-β-D-Manp-(1→
```

Pathogen	Glycan type	Structure	Ref.
C neoformans	CPS	→2)-α-D-Manp-(1→	(Cherniak et al., 1988; Cherniak and Sundstrom, 1994)

```
                    →2)-α-D-Manp-(1→

  β-D-GalpA    β-D-Xylp    β-D-Xylp
      1            1            1
      ↓            ↓            ↓
      2"           2'           2

→3)-α-D-Manp-(1→3)-α-D-Manp-(1→3)-α-D-Manp-(1→
                serotype A

  β-D-GalpA    β-D-Xylp    β-D-Xylp
      1            1            1
      ↓            ↓            ↓
      2"           2'           2

→3)-α-D-Manp-(1→3)-α-D-Manp-(1→3)-α-D-Manp-(1→
      4"
      ↑
      1
   β-D-Xylp

                serotype B

  β-D-GalpA    β-D-Xylp    β-D-Xylp
      1            1            1
      ↓            ↓            ↓
      2"           2'           2

→3)-α-D-Manp-(1→3)-α-D-Manp-(1→3)-α-D-Manp-(1→
      4"          4'
      ↑           ↑
      1           1
   β-D-Xylp    β-D-Xylp

                serotype C

  β-D-GalpA    β-D-Xylp    β-D-Xylp
      1            1            1
      ↓            ↓            ↓
      2"           2'           2

→3)-α-D-Manp-(1→3)-α-D-Manp-(1→3)-α-D-Manp-(1→

                serotype D

Variable 6 O-Acetylation of Man
```

Table 2. – *Continued.*

Pathogen	Glycan type	Structure	Ref.
M. tuberculosis	Cell wall	(structures below)	(Berg et al., 2007)

$[\alpha\text{-D-Man}p\text{-}(1{\to}2)]_{0\text{-}2}\text{-}\alpha\text{-D-Man}p\text{-}(1{\to}2)\text{-}\alpha\text{-D-Man}p\text{-}(1{\to}5)\text{-}$

$\beta\text{-D-Ara}f\text{-}(1{\to}2)\text{-}\alpha\text{-D-Ara}f$
1
↓
5
$\beta\text{-D-Ara}f\text{-}(1{\to}2)\text{-}\alpha\text{-D-Ara}f\text{-}(1{\to}3)\text{-}\alpha\text{-D-Ara}f\text{-}(1{\to}5)\text{-}[\alpha\text{-D-Ara}f\text{-}(1{\to}5)]_2\text{-}\alpha\text{-D-Ara}f$
1
↓
5
$\beta\text{-D-Ara}f\text{-}(1{\to}2)\text{-}\alpha\text{-D-Ara}f\text{-}(1{\to}5)\text{-}[\alpha\text{-D-Ara}f\text{-}(1{\to}5)]_2\text{-}\alpha\text{-D-Ara}f\text{-}(1{\to}3)\text{-}\alpha\text{-D-Ara}f\text{-}(1{\to}5)\text{-}\alpha\text{-D-Ara}f\text{-}$
3
↑
1
$\beta\text{-D-Ara}f\text{-}(1{\to}2)\text{-}\alpha\text{-D-Ara}f$

ManLAM

$[\alpha\text{-D-Man}p\text{-}(1{\to}2)]_2\text{-}\alpha\text{-D-Man}p\text{-}(1{\to}6)\text{-}\alpha\text{-D-Man}p\text{-}(1{\to}6)\text{-}$
2
↑
1
$\alpha\text{-D-Man}p$
LM

$[\alpha\text{-D-Man}p\text{-}(1{\to}6)]_2\text{-}[\alpha\text{-D-Man}p\text{-}(1{\to}6)]_2\text{-}\alpha\text{-D-Man}p\text{-}(1{\to}6)\text{-}myo\text{-Ino-Gro2OR}^1\text{3OR}^2$
2
↑
1
$\alpha\text{-D-Man}p\text{6OR}^3$

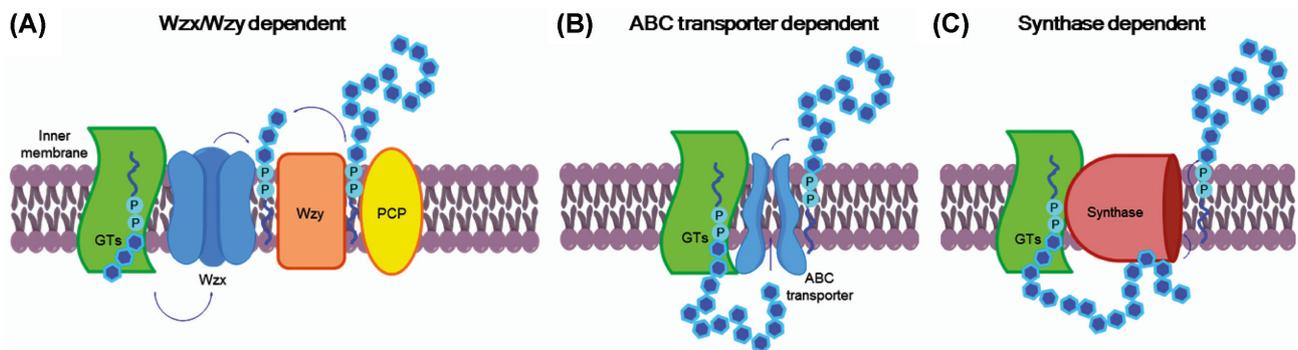$R^1, R^2, R^3 = $ fatty acids
PIMs

Figure 2. Mechanisms for polysaccharide biosynthesis in bacteria. (**A**) In the Wzx/Wzy-dependent pathway, the polysaccharide is built on a undecaprenol diphosphate (UndPP) acceptor, on which cytosolic sugar nucleotides are attached by glycosyltransferase (GT) catalyzed reactions an then exported across the membrane by a flippase Wzx protein for final polymerization by a Wzy polymerase, under the control of a polysaccharide copolymerase (PCP). (**B**) In the ABC transport-dependent pathway, the polysaccharide is built up at the cytoplasmic face of the inner membrane by GTs, and then exported by the ABC transporter. (**C**) In the synthase-dependent pathway, the polysaccharide is assembled at the cytoplasmic face of the inner membrane by a synthase that is also involved in its transportation across the membrane.
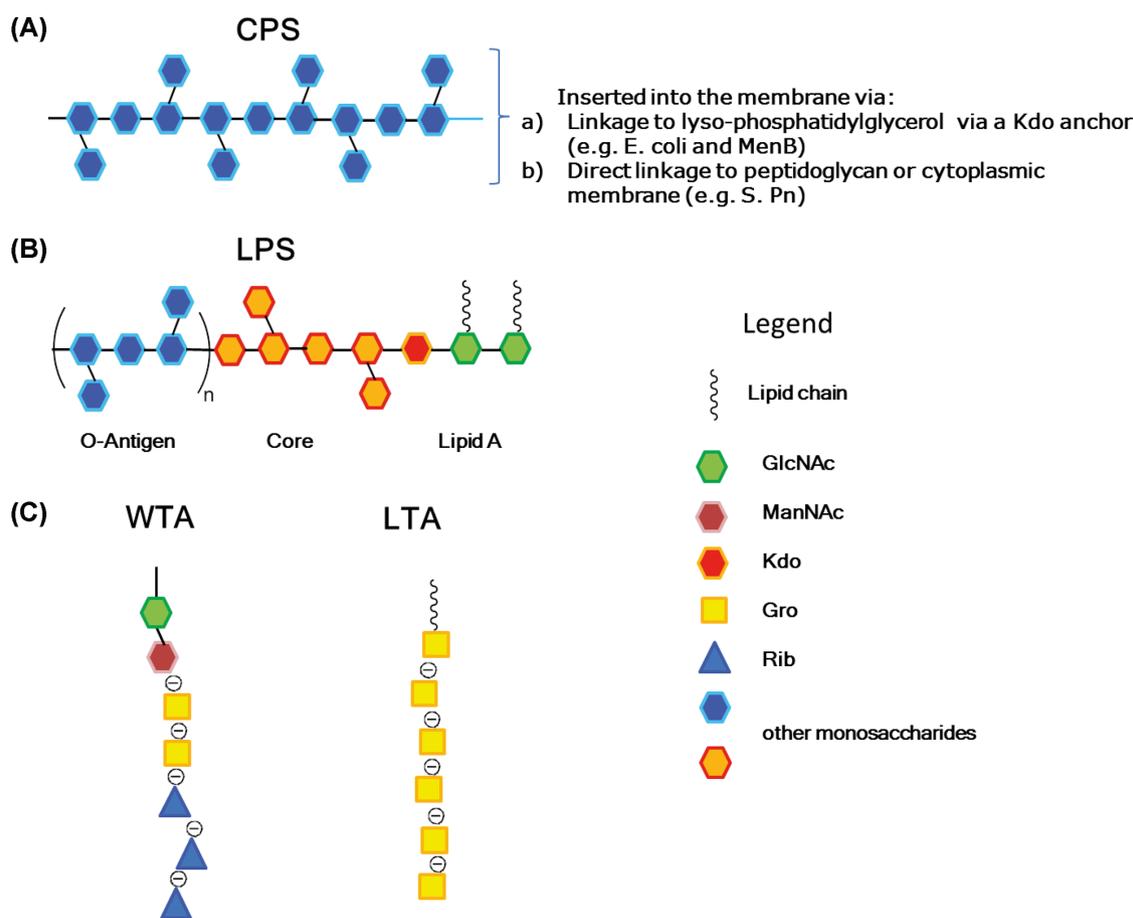

Figure 3. General structures of bacterial surface polysaccharides. (**A**) Capsules are homopolymeric or heteropolymeric carbohydrate chains inserted into the membrane. (**B**) LPS is made of three components: lipid A, core-oligosaccharide and O-polysaccharide or O-antigen. LPS lacking of the O-antigen is termed LOS. (**C**) Teichoic acids are differentiated into lipoteichoic acids (LTA) and wall teichoic acids (WTA).

cross-linked by pentapeptide side chains (Vollmer, Blanot and de Pedro 2008). The PG sacculus is a very large polymer that, because of its rigidity, determines cell shape. The PG layer is much thicker in Gram-positive than in Gram-negative bacteria.

Teichoic and lipoteichoic acids
In Gram-positive bacteria, threading through the layer of peptidoglycans, there are teichoic acids (TA), zwitterionic

glycopolymers containing phosphodiester-linked polyol repeat units (Armstrong *et al.* 1958). TA play crucial roles in cell shape determination, regulation of cell division and other fundamental aspects of Gram-positive bacterial physiology. They are divided into lipoteichoic acids (LTA), which are anchored in the bacterial membrane via a glycolipid, and wall teichoic acids (WTA), which are covalently attached to peptidoglycans (Fig. 1) (Brown, Santa Maria and Walker 2013; Sewell and Brown 2014).

Fully extended membrane-linked LTA may not be able to completely penetrate the PG layer and only reach the bacterial surface once released from the membrane (Reichmann and Grundling 2011). WTA extend through and beyond the cell surface more than LTA do (Silhavy, Kahne and Walker 2010), as confirmed by cryo-EM images for *S. aureus*. WTA are highly abundant modifications of Gram-positive cell walls (Brown, Santa Maria and Walker 2013): in *Bacillus subtilis* and *S. aureus*, for instance, they represent up to 60% of the cell wall (Xia, Kohler and Peschel 2010). WTA are made by two components (Fig. 3C): a disaccharide unit that is highly conserved across bacterial species and a main chain polymer composed of phosphodiester-linked polyol repeating units, generally composed of 1,5-D-ribitol-phosphate (RibP) or (1→3)-L-α-glycerol-phosphate (GroP) (Endl *et al.* 1983; Neuhaus and Baddiley 2003).

Structural diversity of WTA can derive from the presence or absence of substituents attached to the backbone (Fig. 2), including cationic D-alanine esters and a variety of mono- or oligosaccharides, commonly Glc or GlcNAc (Collins *et al.* 2002).

LTA has a simpler and more conserved structure that typically consists of a polyglycerolphosphate (PGP) chain (Fischer, Koch and Haas 1983; Fischer *et al.* 1993) (Fig. 3C). Similarly to WTA, the PGP backbone chain of LTA is modified with D-alanine residues, and in many bacteria with additional glycosyl groups.

Other glycans

Another polysaccharide structure found in many species is the poly-β-D-(1→6)-N-acetylglucosamine (PNAG), a polymer with partial *N*-deacetylation and *O*-succinyl substituents which is one of the major component of biofilms *in S. epidermidis* and *S. aureus* (Joyce *et al.* 2003). Besides cell-to-cell adherence, PNAG also acts as an important virulence factor and protects bacteria against innate host defenses (Little *et al.* 2014).

Synthesis of PNAG in *Staphylococci* is controlled by an operon, icaADBC, codifying for four proteins responsible of biosynthesis and transport across the IM. A similar operon, pgaABCD, has been found in *A. baumannii* as well as in the genomes of a number of Gram-negative bacteria, including *Yersinia pestis*, *Y. enterocolitica*, *E. coli*, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *Burkholderia cepacia*, *P. fluorescens*, *Actinobacillus pleuropneumoniae* and *Aggregatibacter actinomycetemcomitans* (Wang, Preston and Romeo 2004; Tiwary *et al.* 2016), indicating that PNAG is ubiquitous in a number of species.

Mycobacteria and fungal glycans

The architecture of Gram-positive bacteria shares some similarities with mycobacteria and fungi, since they all possess a thick wall outside of their cellular membrane. The cell walls of mycobacteria consist of thin internal layers of peptidoglycans and arabinogalactans, surrounded by a thick layer of micolic acids, glycolipids and cell membrane anchored lipoarabinomannans protruding on the surface. The cell wall of fungi is instead dominated by polysaccharides like mannans (in the form of mannoproteins) and β-(1→3) and β-(1→6) glucans, while a chitin layer is located below (Masuoka 2004; Brown *et al.* 2015; Gow, Latge and Munro 2017).

A particular case is encountered in *Cryptococcus neoformans*, an important cause of meningitis in Africa especially in those categories who are immunocompromised as a consequence of underlying disease like AIDS. *Cryptococcus neoformans* displays a polysaccharide capsule which is essential for its virulence and is composed primarily of glucuronoxylomannan and galactoxylomannan (McFadden, De Jesus and Casadevall 2006).

APPROACHES FOR PRODUCTION OF GLYCOCONJUGATE VACCINES

There are a number of methods to prepare glycococonjugate vaccines: some of them are well established and used in licensed products, others are emerging and increasingly applied to vaccines under development. They are mainly based on covalent linkage between CPS and carrier protein; however, it is worth to mention strategies for non-covalent interaction based on CPS biotinylation followed by association to carrier proteins fused with avidin like peptides (Zhang *et al.* 2013), CPS entrapped in cross-linked protein (Thanawastien *et al.* 2015), and liposomal encapsulation of CPS and proteins (Jones *et al.* 2017). Historical attempts to develop glycoconjugate vaccines based on non-covalent association of CPS with proteins, although promising in animal models, have failed in humans (Anderson *et al.* 1985), thus anticipating an intense validation effort for these new attempts. Below the main approaches for glycoconjugate vaccines are discussed, and some examples are schematically reported in Fig. 4.

Semisynthesis: extraction of polysaccharide and carrier protein moieties from bacteria

The classical approach used for glycoconjugate vaccines is based on polysaccharide extraction from bacterial fermentation, subsequent purification and chemical modification to install a covalent linkage between the saccharide and the carrier protein (Costantino, Rappuoli and Berti 2011; Ravenscroft *et al.* 2015; Khatun *et al.* 2017). The carrier protein is also derived from bacteria by fermentation and subsequent purification and, depending on the chemistry used, it can be conjugated via its functional groups or alternatively derivatized before polysaccharide linkage. Adequate linkers are often used to facilitate the conjugation, reducing steric hindrance between protein and saccharide.

In some cases specific chemical moieties of the polysaccharide, such as the carboxyl group of sialic acid residues, are randomly derivatized and subsequently conjugated to the carrier protein. In some other cases, cis-diols present on the saccharide chain can be modified by $NaIO_4$ oxidation in order to generate aldehydes (Fig. 4B). These chemical groups can be directly linked to the ε-amine of the protein lysine residues by reductive amination, or further derivatized before linkage to the protein (Anderson *et al.* 1986; Marburg *et al.* 1986; Zou and Jennings 2009; CDC 2016). Alternatively, the polysaccharides can be fragmented, for example, by acid treatment, and subsequently sized, by means of chromatography or ultrafiltration, to obtain more defined oligosaccharide populations for protein conjugation (Costantino *et al.* 1999; Broker, Berti and Costantino 2016). In this case the protein coupling is generally carried out using the end reducing sugar which can be modified with a spacer bearing appropriate functional group reacting with the protein (Fig. 4C).

Depending on the polysaccharide structure (e.g. *H. influenzae* type b CPS), $NaIO_4$ oxidation results in simultaneous fragmentation and generation of terminal aldehyde groups available for conjugation. Similarly, treatment of polysialic acids (e.g. *N. meningitidis* serogroup C) with hydrogen peroxide results in depolymerization and concomitant activation through introduction of carbonyl groups (Ryall 2003; Neyra, Paladino and Le
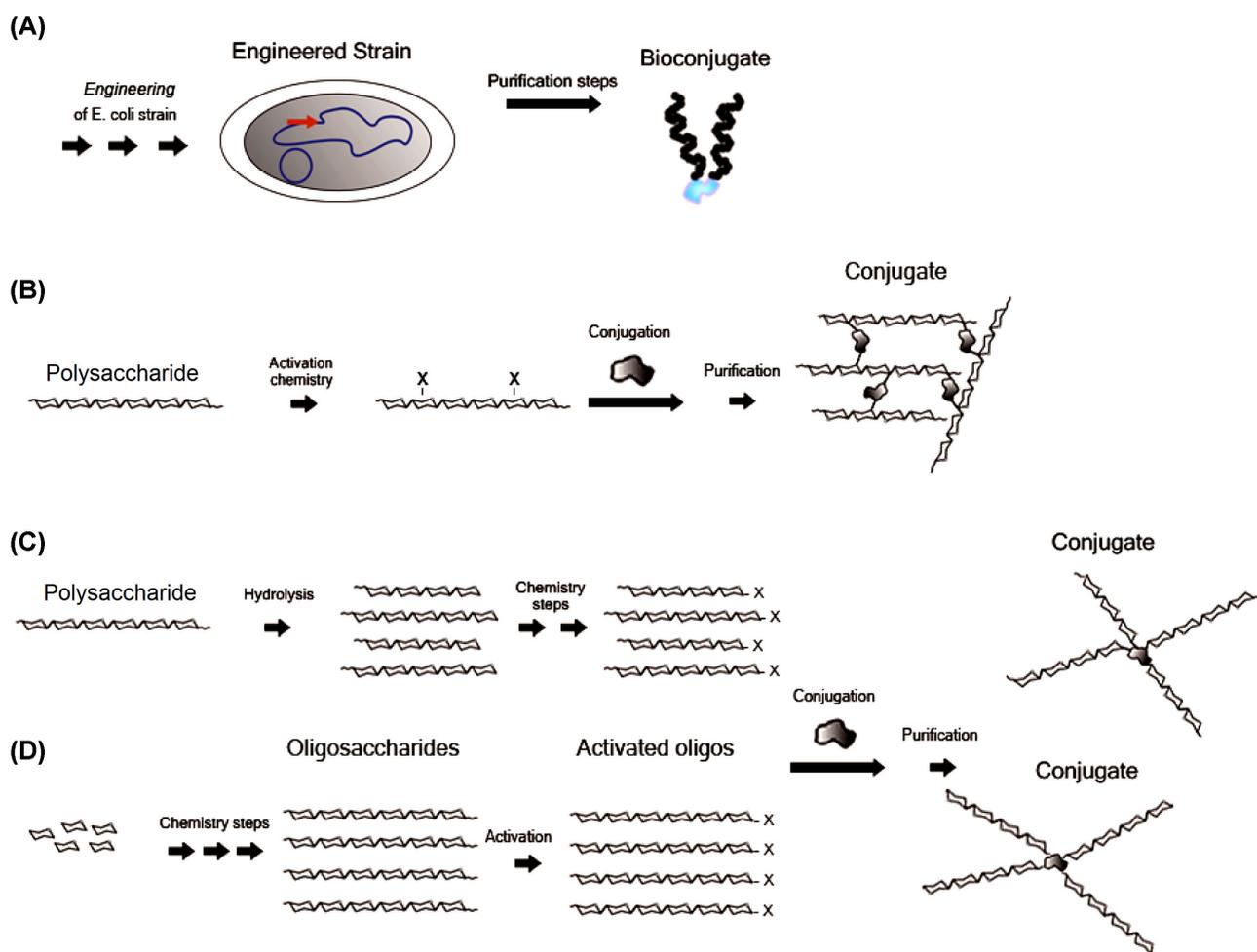
Figure 4. Approaches for the production of glycoconjugate vaccines include (**A**) engineering of *E. coli* for expression of carbohydrate, carrier protein and *in vivo* conjugation, resulting in glycans radially oriented relative to protein; (B) polysaccharides activation of sugar residues along the chain and conjugation to the carrier protein, resulting in cross-linked structures; (C) polysaccharide fragmentation (hydrolysis or other methods discussed in the text), sizing and conjugation via end terminal residues, resulting in glycans radially oriented relative to protein; (D) construction of the oligosaccharide from appropriate building blocks with an in-built linker for conjugation, also resulting in glycans radially oriented relative to protein.

Borgne 2015). Hydrogen peroxide has been used also for depolymerization of *Salmonella* Typhi (Vi CPS) (Arcuri *et al.* 2017).

Many licensed glycoconjugate vaccines to prevent *St. pneumoniae*, *N. meningitidis* and *H. influenzae* caused infections are prepared based on these approaches. Five carrier proteins have been used in these vaccines: tetanus toxoid (TT), diptheria toxoid (DT), CRM$_{197}$, the outer membrane protein complex of meningococcus B (OMPC) and Protein D from *H. influenzae*. For a more technical discussion on this topic, we refer the reader to other reviews (Broker *et al.* 2011, 2017; Costantino, Rappuoli and Berti 2011).

Often the polysaccharides are attached to multiple points of the carrier proteins in a random fashion (Adamo *et al.* 2013). However, recently the possibility to chemically conjugate the glycan at predetermined sites of the carrier protein is emerging (Nilo *et al.* 2015; Stefanetti *et al.* 2015). Also, by means of recombinant techniques, unnatural amino acids with side chains suitable for specific conjugation to the polysaccharide can be inserted in specific regions of the protein sequence (Hu, Berti and Adamo 2016; Kapoor *et al.* 2018). These approaches render the conjugation procedures more selective, allowing for a more rational design of the vaccine and preservation of the key protein epitopes.

Recently, an alternative glycoconjugation method based on dry glycation following periodate oxidation of pneumococcal CPS has been reported (Turner *et al.* 2017).

Synthetic approach: production of the saccharide moiety by means of organic synthesis

The immense progress in the chemical synthesis of carbohydrates seen over the last decades has led to developing protocols for the preparation of a variety of complex bacterial oligosaccharides (Smoot and Demchenko 2009; Morelli, Poletti and Lay 2011) (Fig. 4D).

The climax of this approach has been the development and introduction in Cuban routine vaccination schedule of a conjugate vaccine against *H. influenzae* type b, where the carbohydrate moiety has been obtained by large-scale synthesis of the capsular oligosaccharide (Verez-Bencomo *et al.* 2004). A glycoconjugate based on synthetic carbohydrate of *Sh. flexneri* type 2a has recently entered clinical trials (van der Put *et al.* 2016). Synthetic oligosaccharides are generally prepared with a built-in spacer for conjugation at the downstream end, and offer advantages including the avoidance of handling pathogens, lack of bacterial impurities, minimal batch-to-batch variability and

higher quality control standards during process manufacturing (Adamo 2017).

The introduction of methods for solid phase automated oligosaccharides synthesis (Seeberger 2015; Hahm *et al.* 2017) and automated polymer supported by HPLC-assisted flow chemistry (Ganesh *et al.* 2012) could simplify and accelerate large-scale production of oligosaccharides. A study of the database 'glycoscience.de' showed that a minimal set of 36 monosaccharide building blocks would be sufficient to construct 75% of the catalogued 3299 mammalian oligosaccharides (Werz *et al.* 2007). Despite bacterial glycans are generally characterized by more complex structures than mammalian ones, with numerous branchings and substituents, like acetyl or phosphate groups, this approach has been proven applicable for the fast production of a variety of carbohydrates (Schumann *et al.* 2017).

In addition, other methods, including one-pot protocols (Huang *et al.* 2004; Wu *et al.* 2017) and chemo-enymatic approaches (Wang *et al.* 2013; Fiebig *et al.* 2016; Li and Wang 2016), are accelerating the production of synthetic saccharides rendering their use increasingly attractive, particularly when the polysaccharide is difficult to be purified at high yields, or for the production of more stable sugar mimics (Gao *et al.* 2013).

Bioconjugation approach: engineering *E. coli* for *in vivo* glycoconjugate production

The *in vivo* production of glycoproteins has recently found application in the delivery of a series of glycoconjugate vaccine candidates (Wacker *et al.* 2002). *Escherichia coli* is engineered with genome integrated pathogen glycan clusters, with an oligosaccharyl transferase (PglB) from *Campylobacter jejuni*, integrated in the genome or plasmid encoded, and with a plasmid encoding for the carrier protein with N-glycosylation consensus sequences Asp/Glu-Asn-X-Ser/Thr (where X can be any amino acid except proline) in selected sites (Feldman *et al.* 2005; Kowarik *et al.* 2006; Wacker *et al.* 2006) (Fig. 4A). In detail, pathogen glycans repeating units are expressed in the cytoplasm, assembled onto the *E. coli* lipid carrier UndPP and then flipped across the cytoplasmic membrane. The repeats are polymerized by a polymerase in the periplasmic space, where PglB enables the transfer of the resulting lipid linked oligosaccharides to asparagine residues of the N-glycosylation consensus sequences of the carrier protein.

Biosynthesis of glycoproteins from a number of bacterial polysaccharides, including *Salmonella enterica*, *Shigella* spp, *E. coli* LPS and *S. aureus* serotype 5 or 8 CPS and *St. pneumoniae* CPS has been achieved through this technology (Wetter *et al.* 2012, 2013; Wacker *et al.* 2014; van den Dobbelsteen *et al.* 2016; Ravenscroft *et al.* 2017). Selective glycosylation allows for a better exploitation of proteins with the dual carrier/antigen role, as demonstrated for *S. aureus* α toxin Hla used as carrier for type 5 and 8 CPS (Wacker *et al.* 2014). Phase-1 trials of monovalent vaccines against *Shigella dysenteriae* O1 and *Sh. flexneri* 2a (Hatz *et al.* 2015), and a tetravalent anti-extra intestinal pathogenic *E. coli* (ExPEC) vaccine have been successfully completed (Huttner *et al.* 2017).

Recently, glycoengineering of outer membrane vescicles (ge-OMVs) for expression of heterologous polysaccharides attached to O-Ag negative lipid A core has been proposed as a novel platform for polysaccharide vaccines (Chen *et al.* 2016; Price *et al.* 2016; Valguarnera and Feldman 2017). OMVs combine antigen presentation with optimal size for immune stimulation and proper adjuvant properties for the presence of Toll-like receptors (TLR) 2 and 4. Price *et al.* (2016) showed the efficacy of geOMVs as

vaccines against *St. pneumoniae* in mice, and *Ca. jejuni* in chicken. In another study, *Francisella tularensis* O-Ag expressed on *E. coli* OMVs provided protection against *F. tularensis* (Chen *et al.* 2016).

Cell surface glycans as target for glycoconjugate vaccines

The currently licensed glycoconjugate vaccines (Table 1) were developed based on the epidemiology of bacterial infectious diseases, which registered in the past decades a high incidence of bacterial meningitis caused by *N. meningitidis*, *H. influenzae* type b and *St. pneumoniae* (Pace and Pollard 2007; Pace 2013; Vella and Pace 2015). A number of new glycoconjugate vaccines are being advanced building up on the success of this first generation. Some of them address new pathogens, while others can be considered an extension of current ones to cover additional emerging serotypes.

For example, with the evolving of the pneumococcal epidemiology, non-vaccine serotypes are emerging. Therefore, extension from the current 10- and 13-valent conjugate vaccines (Geno *et al.* 2015; Delgleize *et al.* 2016) up to a 15-valent using CRM$_{197}$ as carrier is under development (McFetridge *et al.* 2015) (Table 1). The increasing medical need in elderly population and the additional emergence of non-vaccine-serotype disease is expected to driving the development of even higher valence vaccines.

Recent outbreaks in Africa (Boisier *et al.* 2007) have highlighted the need for an anti serogroup X meningococcal vaccine (Xie *et al.* 2013), in addition to the already available A, C, W and Y. Conjugates of CPS X (Table 2) (Bundle, Smith and Jennings 1974) were immunogenic and induced bactericidal antibodies in mice (Micoli *et al.* 2013a). Oligomers of various lengths have also been produced by enzymatic and synthetic methods (Morelli *et al.* 2014; Harale *et al.* 2015; Fiebig *et al.* 2016).

Among the novel targets, a recent analysis from WHO (WHO 2017) and CDC (CDC 2013a) highlights bacteria that are increasingly developing resistance to current antimicrobial therapies and are considered an emerging and serious threat for the public health (Garcia-Quintanilla *et al.* 2016). Most of the resistant bacteria belong to the category of nosocomial pathogens, but there are also examples in the community acquired infectious diseases.

In addition to the CPS, other cell surface glycans are being taken into consideration in the development of novel conjugate vaccines. Moreover, along with the classical semisynthetic chemistry, other approaches, including the use of synthetic carbohydrates or *E. coli* glycoprotein expression, are increasingly taking place.

Table 1 reports a list of glycoconjugate vaccines which are at different stage: licensed (L), in clinical trials (C) or discovery (D). Proposed surface glycan antigens and utilized approaches are also included.

Below we describe the more relevant pathogens for which a medical need has been identified and the different classes of microbial glycans (CPS, O-Ag and other surface carbohydrates) that have been targeted for vaccine development.

Capsular polysaccharides

Acinetobacter baumannii

The Gram-negative bacterium *A. baumannii* is the major cause of nosocomial infections and it has frequently been reported in times of war and natural disasters (Fournier and Richet

2006). It affects different human organs, particularly the lungs, causing ventilator-associated pneumonia (VAP) which usually develops to septicemia in intensive care unit residents. Patients at risk are immunocompromised, elderly, premature neonates and patients undergoing surgeries, and its danger has increased due to emerging antibiotic resistance (Peleg, Seifert and Paterson 2008). Recently, a monoclonal antibody against *A. baumannii* K1 capsule was produced and shown protective in a rat challenge model (Russo *et al*. 2013). The K1 CPS (Table 2) was also demonstrated as a potential vaccine antigen via passive immunization. However, an anti K1 CPS monoclonal antibody (mAb) recognized only 13% of the tested strains, and there is little information on the prevalence in clinical isolates (antigen epidemiology) of the nearly 40 serovars identified so far, which complicate the development of a CPS-based vaccine (Chen 2015).

Burkholderia pseudomallei and mallei

Burkholderia pseudomallei (Bp) is a Gram-negative saprophyte that causes melioidosis. It is highly resistant to harsh environmental pressures, and it is classified as a potential class B bioterrorism weapon due to its high infectivity when aerosolized (Silva and Dow 2013; Peacock *et al*. 2012). The intrinsically high resistance of Bp to several different classes of antibiotics increases the potential danger of this organism. Melioidosis is acquired by skin inoculation, inhalation and ingestion, with pneumonia being the most common clinical presentation. This disease is prevalent in South-East Asia and Northern Australia, and persons with open skin wounds and those with diabetes or chronic renal disease are at increased risk for this infection, particularly among individuals performing agricultural work without health care standard protections.

Bp possesses a CPS (named O-polysaccharide I, OPSI) which was originally identified as an LPS (Knirel *et al*. 1992). OPSI is composed of linear (1→3)-linked 2-O-acetyl-6-deoxy-β-D-manno-heptopyranose residues with O-acetylation at position 2 (Table 2), and it is required for serum resistance and virulence (Perry *et al*. 1995). Similarly to Bp, *B. mallei* (Bm), the causative agent of glanders, expresses only this single serotype of capsule (DeShazer, Brett and Woods 1998; DeShazer *et al*. 2001; Nelson *et al*. 2004). Therefore, a CPS-based vaccine would potentially offer cross-protection against both pathogens (Scott *et al*. 2014). CPS-BSA conjugates were immunogenic in mice and elicited antibodies protective against infection and opsonic (Nelson *et al*. 2004). A synthetic hexasaccharide of Bp capsule coupled to the Hc domain of TT elicited anti-CPS antibodies in mice that were protective against Bp infection (Scott *et al*. 2016).

Enterococcus faecalis and faecium

Until the late 1970s, Gram-positive enterococci were considered a relatively inoffensive group of pathogens against which effective antibiotics were readily available. Emergence of antibiotic resistance and increased isolation from hospitalized patients, where they account for 11% of nosocomial bloodstream isolates, have pointed out the relevance of Enterococci as nosocomial pathogens (Theilacker *et al*. 2004), overtaken in terms of epidemiology only by *S. aureus*. It is estimated that in the USA about 66 000 enterococcal infections occur each year, and about 20 000 of these are due to multiple-drug-resistant strains, with about 1300 deaths per year (Reyes, Bardossy and Zervos 2016).

Initially, a CPS with an LTA-like structure composed of α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)-Gro-3P (Table 2) was isolated in *En. faecalis* (Huebner *et al*. 1999; Wang *et al*. 1999). A different surface-exposed polysaccharide composed of glucose, galactose,

glycerol and phosphate in a 4:1:1:2 ratio was also reported (Hancock and Gilmore 2002). Based on the analysis of the biosynthetic CPS locus, four serotypes A-D were later described (Hufnagel *et al*. 2004). A recent analysis of clinical *En. faecalis* isolates indicated that most pathogenic strains belong to serotype C. This suggested that a limited number of *En. faecalis* capsular serotypes would be needed for developing a broadly active immunotherapeutic agent. However, in another study, a collection of 157 isolates was examined, and only half of them could be typed to any of these four serotypes indicating that serotype diversity might be larger (Hufnagel *et al*. 2006).

Early studies showed that purified CPS depleted the opsonic killing activity of immune rabbit sera, and elicited in rabbit high titers of antibodies mediating opsonic killing of bacteria (Huebner *et al*. 1999, 2000). Approximately one-third of a sample of 15 *En. faecalis* strains and 7 vancomycin-resistant *En. faecium* strains were shown to possess shared CPS, target of opsonophagocytic antibodies. CPS also elicited protective antibodies in a mouse model of systemic enterococcal infection (Huebner *et al*. 2000). Anti-CPS antibodies made in rabbits passively protected mice against serologically related enterococcal strains. Furthermore, capsule producing strains of serotype C and D resulted more resistant to complement-mediated opsonophagocytosis than unencapsulated strains (Thurlow *et al*. 2009), supporting the role of the CPS as virulence factor and possible vaccine antigen.

Group B Streptococcus

Group B *Streptococcus* (*Streptococcus agalactiae*; GBS) is an encapsulated Gram-positive β-hemolytic pathogen, leading cause of neonatal sepsis and meningitis (Le Doare and Heath 2013). Risk factors for developing invasive GBS include maternal GBS vaginal-colonization, prematurity, prolonged rupture of membranes (>18 h), chorioamnionitis, young maternal age, black race and having a previous infant with invasive GBS disease. Current strategy for prevention of GBS infection in newborns is centered on maternal vaccination with CPS conjugates (Heath 2016). Since, based on the structure of the CPS structure, 10 serotypes can be differentiated, 5 of which are responsible for the majority of the epidemiology, multiple polysaccharides are used for the development of conjugate vaccines. GSK has sponsored phase-1 and -2 trials of an investigational trivalent (Ia, Ib, III) CPS-CRM$_{197}$ conjugate vaccine, and is currently pursuing pre-clinical studies of a pentavalent (Ia, Ib, II, III, V) CPS-CRM$_{197}$ vaccine (Kobayashi *et al*. 2016). Pfizer has recently announced to enter clinical trials with a multivalent formulation (Kobayashi *et al*. 2016).

Haemophilus influenzae type a

Among the six different capsulated strains (a–f) of the Gram-negative *H. influenzae,* b and a are the most infective ones. Hib was the first pathogen against which a conjugated CPS vaccine was developed and introduced in vaccination schedules, with consequent significant decrease of incidence. In recent years, increasing rates of invasive infection due to Hia have been reported in Canada, Alaska, Aboriginal populations in southwestern USA and Australia, and in Brazil (Ribeiro *et al*. 2003; Boisvert and Moore 2015). However, due to lack of comprehensive surveillance programs in many countries, the epidemiological data of Hia-associated diseases are neither complete nor accurately recorded, potentially underestimating the impact of Hia infection worldwide (Ulanova and Tsang 2014). Cases of non-Hib disease have been reported to exhibit AMR to commonly used therapeutic agents making treatment more challenging (Skaare *et al*. 2014). Hia and Hib share a similar CPS structure (Table 2), but

no cross-protection is afforded to type a by immunization with Hib conjugate vaccines (Jin *et al.* 2007). Considering the similarity of the two CPS, a conjugate vaccine against Hia is likely to be effective and the development of a vaccine comparable to the current Hib conjugate appears reasonable (Boisvert and Moore 2015). This hypothesis was corroborated by recent conjugation of sized and activated Hia polysaccharide to CRM$_{197}$ and protein D as carriers (Cox *et al.* 2017). The glycoconjugates were immunogenic in rabbits and elicited bactericidal antibodies.

Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative pathogen belonging to the family of *Enterobacteriaceae,* implicated in severe infections and outbreaks with high mortality especially for multidrug-resistant infections (Brady *et al.* 2016). In 2013, it was reported among the top five causes of hospital-acquired infection in EU (Suetens *et al.* 2013) and the second leading cause of Gram-negative blood stream infection. Depending on the type of infection and the mode of infectivity, cells of *Klebsiella* spp. may adhere and attack upper respiratory tract epithelial cells, cells in gastrointestinal tract, endothelial cells or uroepithelial cells, followed by colonization of mucosal membranes. Common underlying conditions include alcoholism, diabetes mellitus, chronic liver disease (cirrhosis), chronic renal failure, cancer, transplants, burns and/or use of catheters. *Klebsiella* spp. can be transmitted through skin contact with environmentally contaminated surfaces and/or objects, and less frequently by fecal transmission (Janda and Abbott 2006). Seventy-eight capsular antigens (K antigens), leading to different serogroups, have been identified, although about 24/25 were reported to cover about 70% of epidemiology (Podschun and Ullmann 1998). The role of the capsule as virulent factor was demonstrated by pioneering studies by Cryz *et al.* (Cryz 1983; Cryz, Furer and Germanier 1984). Strains with capsular serotypes K1 and K2 (Table 2) have been identified as the predominant virulent strains, and their virulence has been confirmed in mouse models (Struve *et al.* 2015).

The capsules of *K. pneumoniae* are complex acidic polysaccharides (CPS) consisting of repeating units composed of four to six sugars, one of which is often an uronic acid (Corsaro *et al.* 2005). The synthetic hexasaccharide repeating unit of the capsule from carbapenem-resistant strains belonging to the sequence type 258 (ST258), found in some isolates in the USA and Israel (Diago-Navarro *et al.* 2014), has recently been demonstrated to bind a specific mAb and to promote, after conjugation, the production of phagocytic antibodies (Seeberger *et al.* 2017).

A 24-valent CPS-based vaccine passed phase 1 in human trials, but the maximum protection coverage never exceeded 70% of the *K. pneumoniae* strains (Ahmad *et al.* 2012a). Consequently, attention has been addressed to other surface polysaccharides, particularly the O-Ag, which will be discussed later.

Salmonella species

Salmonella enterica serovar Typhi (*S.* Typhi), which causes the so-called typhoid fever, is still a major problem in low-income countries, such as South and South-East Asia, affecting millions of people each year (Mogasale *et al.* 2014). Vi CPS (Table 2) is currently licensed as a vaccine against typhoid fever (MacLennan, Martin and Micoli 2014). However, being an T-independent antigen, Vi is not immunogenic in infants and is only licensed for children over 2 years of age (Lebacq 2001). While a phase 3 study was reported more than 15 years ago demonstrating high protective efficacy of Vi CPS conjugated to *r*EPA (Lin *et al.* 2001), only recently Vi-TT and Vi-*r*EPA conjugate vaccines were licensed in India and China (MacLennan, Martin and Micoli 2014). CPS-based glycoconjugate vaccines against *S.* Typhi are currently under development by a number of manufacturers (Table 1).

Staphylococcus aureus

Among the Gram-positive bacteria, staphylococci account for a large proportion of hospital-acquired infections (Theilacker *et al.* 2004). High rates are observed for methicillin-resistant *S. aureus* infections (MRSA), which cause mostly pneumonia, skin-, wound-, bloodstream- and surgical site infections (Theilacker *et al.* 2004). In the USA, the annual incidence of *S. aureus* bacteremia is of 15–17 cases per 100 000 population, of which nearly half are due to MRSA (Hidron *et al.* 2008), justifying the need for vaccination. Although there are at least 12 capsular types, CPS5 and 8 (Jones 2005) (Table 2) comprise ∼85% of blood infections, and their use for vaccine development was explored (Robbins *et al.* 2004). A single unadjuvanted dose of the bivalent vaccine composed of *S. aureus* CPS5 and 8, bound to *r*EPA, showed a trend of efficacy over the first 40 weeks postvaccination (Fattom *et al.* 2004a,b). However, the same vaccine did not show benefit compared to placebo when tested in further trials in end-stage renal disease patients as target population (Fattom *et al.* 2015). CPS5 and 8 conjugated to TT, in combination with mutated detoxified alpha-toxin and clumping factor A (ClfA), with and without the adjuvant AS03B, were safe and induced a strong humoral response in a phase-1 clinical trial with healthy adults conducted by GSK (Levy *et al.* 2015). Also CRM$_{197}$ conjugates of CPS5 and 8 in mixture with ClfA and manganese transporter C, without adjuvant, were well tolerated and immunogenic in a phase-1 clinical trial conducted by Pfizer (Nissen *et al.* 2015; Frenck *et al.* 2017).

O-Antigens

O-Ag components of LPS molecules have been recognized as virulence factors and suggested as potential vaccine candidates for different pathogens.

Burkholderia pseudomallei and mallei

LPS from Bp, generally referred as OPSII, is genetically related and structurally similar to the one from Bm. The O-Ag structure consists of a linear heteropolymer of a disaccharide composed of β-D-glucopyranose (1→3)-linked to 6-deoxy-α-L-talopyranose (Table 2). While some studies have focused on the OPSI capsule as unique antigen for a vaccine, others indicate OPSII to be required for serum resistance and virulence (DeShazer, Brett and Woods 1998; DeShazer *et al.* 2001). LPS-specific monoclonal antibodies were proven passively protective in animal models of infection (Treviño 2006; AuCoin 2012). Interspecies variations within the O-Ag lie in the different substitutions of the 6-deoxytalose residues, particularly O-acetylation at both C4 and C2 and O-methylation at C2 (Brett, Burtnick and Woods 2003). O-Acetylation at the C4 position has been detected in significant amounts in Bp, whereas it is absent in Bm strains (Heiss *et al.* 2013). In a recent study, among a panel of seven disaccharides variably substituted, the disaccharide with a 2-O-acetylated-3-O-methylated 6d-Tal unit showed the best binding to a LPS-specific mAb, known to be passively protective in mouse models of melioidosis and glanders, and gave the highest anti-LPS immune response after conjugation to CRM$_{197}$ (Kenfack *et al.* 2017). A bioconjugate of OPSII with a *Campylobacter* protein AcrA was shown to be immunogenic in mice and moderately increased protection of mice after intranasal challenge (Garcia-Quintanilla *et al.* 2014).

Escherichia coli

Escherichia coli is a Gram-negative bacterium that can be broadly classified as either diarrheagenic or extra-intestinal pathogenic *E. coli* (Croxen and Finlay 2009). ExPEC cause a broad variety of infections including urinary tract infections (UTI) and bacteremia, an increasing problem in the aging population (Russo and Johnson 2003). The emergence of antibiotic-resistant strains has resulted in increased numbers of hospitalizations for UTI, high risk of death in patients with bacteremia and intensified treatment costs (Zilberberg and Shorr 2013). O-Ag specific antibodies confer protection against *E. coli* infections (Sarkar *et al.* 2014). The immunogenicity and safety of a tetravalent ExPEC vaccine produced by the process of bioconjugation, composed of the O1, O2, O6 and O25b antigens (Table 2) linked to *rEPA*, showed good immunogenicity in animal models (van den Dobbelsteen *et al.* 2016). This bioconjugate vaccine candidate, co-developed by Limmatech Biologics AG and Janssen Pharmaceuticals Inc., was well tolerated and elicited functional antibody responses against all vaccine serotypes in women with a history of recurrent UTI (Huttner *et al.* 2017).

Francisella tularensis

Francisella tularensis is a highly-infectious Gram-negative bacterium that causes the rapid, and often lethal disease, tularemia (Rowe and Huntley 2015). It has been classified by the Center for Disease Control and Prevention as a category A bioweapon (Dennis *et al.* 2001). Humans can acquire this infection through several routes including a bite from an infected tick, deerfly or mosquito, contact with an infected animal or its dead body, drinking contaminated water and breathing contaminated dirt or aerosol. Clinical manifestation of the disease is dependent on the biotype, inoculum and port of entry (Ulu-Kilic and Doganay 2014).

A key factor in the biology of this bacterium is LPS, which poorly activates proinflammatory responses due to its lack of interaction with TLR4. LPS molecules can be modified by various carbohydrates, including Glc, Man and GalNAc, affecting various aspects of virulence. Mutants devoid of O-Ag (Table 2) show reduced intracellular survival and mouse virulence. The inability of the LPS to alarm the immune system coupled with its frequent modification/alteration likely aid the success of this pathogen during human infection (Gunn and Ernst 2007). An OAg-*rEPA* bioconjugate was successfully produced (Cuccui *et al.* 2013) and resulted able to boost IgG levels and significantly increase the time to death upon subsequent challenge with *F. tularensis*. The inner core region of the LPS of *F. tularensis* was synthesized and proved to be recognized by antiserum against LPS and a live vaccine strain, supporting to further explore this compound as a vaccine candidate (Boltje *et al.* 2012). O-Ag displayed on OMVs from a hyperblebbing *E. coli* strain induced high levels of specific IgG titers, as well as vaginal and bronchoalveolar IgA antibodies, and provided protection against challenge with *F. tularensis* strain (Chen *et al.* 2016).

Klebsiella pneumoniae

In contrast to the large number of capsular serotypes, *K. pneumoniae* has only nine LPS O groups, and in a recent study serotypes O1, O2 and O3 accounted for 80% of infections (Follador *et al.* 2016). The O-Ag is accessible to antibodies in encapsulated strains (Rukavina *et al.* 1997; Ahmad *et al.* 2012a). However, it is unclear whether this is true for most of clinical isolates, since in some K serogroups O-Ag appears masked by CPS (Tomás *et al.* 1991).

Conjugate vaccine of the O-Ag from *K. pneumoniae* M 10 and iron-regulated cell surface proteins of the same organism was found immunogenic and protective against challenge in a rat lobar pneumonia model (Chhibber and Bajaj 1995). Immunization of rats with an O-Ag TT conjugate decreased bacterial colonization in lungs, and resulted in activation of alveolar macrophages capable of bacterial phagocytosis *in vitro* (Chhibber, Rani and Vanashree 2005).

The O1 O-Ag chemically linked to *Klebsiella* OM proteins elicited immunoglobulins against different *Klebsiella* infections, which were transferred via placenta to the offspring of the vaccinated rabbits (Ahmad *et al.* 2012b). A non-toxic and immunogenic form of *K. pneumoniae* LPS was obtained by incorporation of the native preparation into liposomes (Chhibber, Wadhwa and Yadav 2004).

O1 and O2 O-Ag share a similar polygalactose structure, termed D-galactan-I, except that O1 is shielded by the outer repeating units (D-galactan-II, Table 2) (Whitfield *et al.* 1991,1992; Vinogradov *et al.* 2002).

Recently, it was found that the vast majority of ST258 isolates, a globally disseminated drug-resistant nosocomial strain, express a modified D-galactan-I O-Ag, termed D-galactan-III (Szijarto *et al.* 2016). Since 83% of the more than 200 ST258 draft genome sequences currently available carry the corresponding operon, these isolates are predicted to express D-galactan-III antigens. Accordingly, a D-galactan-III specific mAb was produced, showing to bind to extracted LPS from a panel of ST258 isolates, irrespective of the distinct capsular antigens expressed. Based on these data, the D-galactan-III antigen may represent an attractive target for immunization approaches against *K. pneumoniae* ST258.

Conjugates of polysaccharides from different serovars (Table 1) to *P. aeruginosa* flagellin have been proven to induce protective antibodies (Simon, Cross and Tennant 2016). Attempts to target common motif in bacterial LPS such as the core tetrasaccharide Hep_2Kdo_2 have been recently made. The structure was synthesized and covalently attached to DT. Rabbit serum elicited against the conjugate was reactive to *N. meningitidis* strains as well as *E. coli* strain St1052 (W3110) and *P. aeruginosa* serotype O6 reference strain (St4017). The serum enabled *N. meningitidis* bacterial killing, when combined to an inhibitor of CPS transport (Kong *et al.* 2016).

Moraxella catarrhalis

Moraxella catarrhalis, also known as *Branhamella catarrhalis*, and previously known as *Neisseria catarrhalis* or *Micrococcus catarrhalis*, is a Gram-negative, aerobic diplococcus, frequently found as a commensal of the upper respiratory tract, particularly in children.

Besides being recognized as the primary cause of acute otitis media after *St. pneumoniae* and *H. influenzae* (Enright and McKenzie 1997), *M. catarrhalis* is also implicated as a pathogen in bronchitis, sinusitis and laryngitis in adults and children and is a major cause of bronchopneumonia and exacerbation of existing chronic obstructive pulmonary disease (COPD) in elderly patients and long-term heavy smokers with chronic pulmonary disease (Sethi and Murphy 2001). It can also cause nosocomial infection, particularly in respiratory, pediatric and intensive-care units (Richards *et al.* 1993). The clinical management of patients infected with *M. catarrhalis* relies predominantly on antimicrobial agents, and growing global emergence of β-lactamase-producing strains (Verduin *et al.* 2002) has highlighted the need for vaccination (Kaieda *et al.* 2005).

The LPS from *M. catarrhalis* lacks of a full-length O chain, and therefore the LOS is a possible virulence factor in the pathogenesis of human infections (Fomsgaard *et al.* 1991). Serological studies have identified three *M. catarrhalis* LOS types (Table 2): A, B and C, representing 61%, 29% and 5%, respectively, of the 95% of the total 302 isolates tested from different geographic locations (Vaneechoutte *et al.* 1990). The inner core is conserved among the three serotypes, while the difference lies in the diversity of their oligosaccharide branches (Edebrink *et al.* 1994, 1995, 1996). Therefore, this LOS might be a good candidate for a vaccine antigen (Verduin *et al.* 2002). Sera from lower respiratory tract-infected patients recognized *M. catarrhalis* LOS (Rahman *et al.* 1995), and convalescent sera from COPD patients possessed IgA antibodies against this molecule (Murphy *et al.* 2005).

Recent studies identified a mAb recognizing a common LOS epitope and facilitating complement killing of *M. catarrhalis* strains from all three major serotypes (Gergova *et al.* 2007). Immunization with detoxified LOS A, B and C coupled to carrier proteins produced in mouse and rabbit models sera inducing complement-mediated bactericidal activity against homologous and some heterologous strains (Gu *et al.* 1998; Yu and Gu 2005, 2007). Mouse antisera elicited by detoxified LOS conjugated to OMP CD or to UspA proteins showed high titers of specific anti-LOS antibodies, with complement-dependent bactericidal activity toward *M. catarrhalis*. In addition, mice immunized with both conjugates showed a significant enhancement of the clearance of *M. catarrhalis* from lungs compared with control mice (Hu *et al.* 2000, 2004).

Neisseria gonorrhoeae

Neisseria gonorrhoeae is the causative agent of the sexually transmitted disease gonorrhea. WHO reports an estimated global incidence of over 106 million cases per year, with a 21% increase in incidence having occurred between 2005 and 2008 (WHO 2012; Edwards *et al.* 2016). A 11% increase in the number of cases has been reported in the USA from 2009 to 2013 (CDC 2013b) and a 90% increase in Australia from 2009 to 2014 (NNDSS 2015). Incidence is likely underestimated due to inadequate surveillance and diagnostics methods in many regions, as well as the high number of asymptomatic cases. Gonococcus has developed resistance to all classes of antibiotics used to treat it over the past seven decades, including the sulphonamides, penicillins, tetracyclines, macrolides and quinolones (Unemo 2015). As other *Neisseria* species, gonococcus biosynthesizes a core LPS pentasaccharide (Table 2), of which extensions from the LOS core heptoses (HepI and HepII) are phase variable. The mAb 2C7 which attenuates gonococcal burden in the mouse vaginal colonization model is directed to LOS. Sugar motifs responsible for total, partial or no complement-dependent killing by mAb 2C7 have been identified (Yamasaki *et al.* 2010; Chakraborti *et al.* 2016). Heptose-monophosphate (HMP) found in *N. gonorrhoeae* core LOS was found as the link between gonorrhea and HIV, since it activates CD4 + T cells to invoke an NF-κB–dependent transcriptional response that drives HIV-1 expression and viral production (Malott *et al.* 2013). The 2C7 epitope is a conserved oligosaccharide (OS) structure expressed by 94% of gonococci that reside in the human genital tract and by 95% of first passaged isolates (Gulati *et al.* 1996).

A peptide mimic (called PEP1) as an immunological surrogate of the 2C7-OS epitope and reconfigured into a multi-antigenic peptide (MAP1) was investigated. Mice immunized with MAP1 developed a Th1-biased anti-LOS IgG antibody response that was also bactericidal, resulting in reduction of the carriage length (Gulati *et al.* 2013).

Non-typeable Haemophilus influenzae

Non-typeable *Haemophilus influenzae* (NHTi) strains lack the polysaccharide capsule, and their virulence is associated with multiple factors, including LOS. So far, the only known natural habitat of *H. influenzae* is the human respiratory tract. The hallmark of NTHi is heterogeneity, and this has been the major obstacle for developing a successful vaccine. *H. influenzae* cause a wide spectrum of diseases ranging from respiratory tract infections to severe invasive disease, such as meningitis, sepsis, bacteraemic pneumonia and epiglottitis (Cerquetti and Giufre 2016). After introduction of Hib vaccination, a marked change in the predominant invasive serotype from Hib to NTHi has taken place. Invasive NTHi disease occurs across all age groups and account for 77% of all notified invasive *H. influenzae* cases in Europe. NTHi is also the most frequently isolated bacterial pathogen in otitis media and sinusitis in children (Murphy, Bakaletz and Smeesters 2009). Acute exacerbations in COPD in adults are almost exclusively associated with NTHi isolates (Soriano and Lamprecht 2012). NTHi colonization/infection is also quite common in young children with cystic fibrosis (CF). In developing countries, NTHi is the major cause of hearing loss, affecting an estimated 65 million to 300 million people globally (Murphy 2015).

Vaccines to prevent otitis media and COPD will have a broad impact in reducing antimicrobial use and resistance (Murphy 2015).

NTHi LOS is structurally and antigenically heterogeneous. To date, 10 serotypes have been identified (Campagnari *et al.* 1987; Patrick *et al.* 1987). Detoxified LOS (dLOS) conjugated to proteins resulted immunogenic in mice and rabbits and conferred T-cell-dependent immunological protection against otitis media in chinchillas (Gu *et al.* 1996, 1997; Sun *et al.* 2000). Intranasal immunization with a dLOS-TT conjugate elicited LOS-specific mucosal and systemic immunity, which enhanced not only the homologous but also heterologous bacterial clearance in mouse nasopharynx (Hirano *et al.* 2003). NTHi OM protein P6 was evaluated as carrier for dLOS, due to its conservation and potential to elicit bactericidal antibodies (Wu *et al.* 2005). Animal studies revealed that P6 could serve as an effective carrier for dLOS.

Peptides that mimic NTHi LOS, conjugated to KLH, were able to induce anti-LOS antibodies in rabbits (Hou and Gu 2003; Balakrishnan 2017). Passive immunization with the anti-LOS sera resulted in enhanced pulmonary bacterial clearance in a mouse model that could be eliminated after pre-absorption of the sera with LOS.

Pseudomonas aeruginosa

Pseudomonas aeruginosa (PA) is a Gram-negative, ubiquitous bacterium, capable of both aerobic and anaerobic growth that can survive on minimal nutritional requirements and tolerate harsh physical conditions, persisting in both community and hospital settings. Serious infections with PA are predominantly hospital acquired (Sharma, Krause and Worgall 2011) and PA has highest mortality rate (37%) of nosocomial infections (Klevens, Edwards and Gaynes 2008; Lister, Wolter and Hanson 2009).

PA may cause fulminant and acute VAP (Klompas *et al.* 2011; Sandiumenge and Rello 2012), be a colonizer in COPD or cause a chronic infection in CF patients (Sharma, Krause and Worgall 2011), with slowly progressive deterioration of pulmonary function as well as non-CF bronchiectasis and COPD patients (Kraemer *et al.* 2005; Veesenmeyer *et al.* 2009).

Antibiotic resistance of PA is a major concern. Antibiotics can alter the bacterial flora in the upper respiratory tract, favoring colonization of resistant nosocomial pathogens and subsequent

pneumonia. Treatment of infections can be very challenging, since most PA are resistant to at least one of the classes of antibiotics, and a few PA are resistant even to all of the antibiotics available (Talbot *et al.* 2006; Pier 2007).

At least 20 different O-Ag structures can be distinguished, although only about 11 of these are expressed in the majority of clinical PA isolates. The lipid A shows variation from CF to bronchiectasis patients in the acylation pattern. Also O-Ag shows variability, as transition to chronic infections is correlated with changes in LPS from smooth (with O-Ag) to rough (with no O-Ag). The core structure seems to remain identical. The most commonly isolated serotypes in acute infection are O1, 6 and 11 (Table 2), yet there is a quote of isolates not expressing O-Ag (Jennings *et al.* 2015).

A heptavalent vaccine prepared from LPS of seven different serotypes, called Pseudogen, showed efficacy in preventing fatal PA infections in non-randomized trials among adult cancer and burn patients, but these vaccines were limited by toxicity and showed no benefit moreover in studies with leukemia and CF patients (Alexander, Fisher and MacMillan 1971; Haghbin, Armstrong and Murphy 1973; Young, Meyer and Armstrong 1973; Pennington *et al.* 1975; Hortobagyi *et al.* 1978). When Pseudogen was tested in CF patients already infected with PA, the patients did clinically worse compared with non-vaccinated controls, perhaps due to exacerbation of inflammation engendered by vaccination (MacIntyre, McVeigh and Owen 1986). An improved LPS-based polyvalent vaccine (16 strains) was investigated in naïve CF patients. The vaccine failed to reduce the rate of PA colonization when compared with the non-vaccinated control group. The same vaccine was also tested in burn patients with inconclusive results (Jones, Roe and Gupta 1978; Langford and Hiller 1984). Possibly, the vaccine did not protect against a sufficient range of PA LPS serotypes (Doring and Pier 2008; Priebe and Goldberg 2014). Aerugen, an 8-valent vaccine from O-Ag conjugated to exotoxin A (EPA), was initially demonstrated safe and immunogenic in plasma donors, bone marrow transplant and non-colonized CF patients (Cryz *et al.* 1987, 1989; Schaad *et al.* 1991; Lang *et al.* 2004). In a small open study involving 30 non-colonized CF, the vaccine was confirmed well tolerated and induced antibodies to the O-Ag promoting the opsonophagocytic killing which were maintained up to 3 years (Cryz *et al.* 1994). In a cohort of 25 CF patients, yearly vaccinations over 10 years induced IgG levels lower than infection-induced IgG titers, but affinity and epitope specificity rather than the quantity of the antibodies was shown to mediate protection (Zuercher *et al.* 2006). However, in a larger trial in European CF patients, Aerugen showed good safety but no significant differences from the placebo (http://www.biospace.com/News/crucell-n-v-announces-suspension-of-aerugenr/24447). In bronchiectasis patients, high titers of IgG2 specific for the O-Ag resulted in impaired PA serum-mediated killing (Wells *et al.* 2014), which could explain the inconsistent results of LPS-based approaches.

Salmonella species

Due to the lack of CPS, the development of O-Ag based vaccines is in progress for *S.* Paratyphi A, and non-typhoid *Salmonella* (NTS). In Asia, a significant proportion of enteric fever is caused by *S.* Paratyphi A, while NTS, mainly *S.* Typhimurium and *S.* Enteritidis, is major cause of bloodstream infection in sub-Saharan Africa (MacLennan, Martin and Micoli 2014). O-Ag from three of the principal invasive serovars (namely, O:2 for *S.* Paratyphi A, and O:4,5 for *S.* Typhimurium and O:9 for *S.* Enteritidis) have been conjugated to carrier proteins and tested in animal models (MacLennan, Martin and Micoli 2014). Conjugation of S. Typhi

O-Ag to rEPA has also been carried out to cover Vi-negative strains (Salman *et al.* 2017). However, no vaccine is yet available against these diseases.

Shigella species

Shigella species have recently been reviewed (Mani, Wierzba and Walker 2016). Shigellosis is caused by the ingestion of bacteria of the genus *Shigella*, of which three species are responsible for the majority of infections. *Shigella flexneri* is the most frequently isolated species worldwide, accounting for most cases in the least-developed countries; *Sh. sonnei* is more common in low- and middle-income countries; and *Sh. dysenteriae* has historically caused epidemics of dysentery, particularly in confined populations such as refugee camps (Mani, Wierzba and Walker 2016). Immunity to *Shigella* appears to be strain-specific, so an O-Ag-based vaccine covering the most commonly detected strains (i.e. *Sh. flexneri* 2a, 3a, 6 and *Sh. sonnei*) is desirable.

A conjugate vaccine composed of *Sh. sonnei* O-Ag bound to *r*EPA conferred type-specific protection against *Sh. sonnei* shigellosis when tested in Israeli army recruits (Cohen *et al.* 1997). *Shigella dysenteriae* type 1 and *Sh. flexneri* 2a bioconjugates (of which the glycan component is depicted in Table 2) elicited significant LPS specific humoral responses in phase-1 studies (Hatz *et al.* 2015; Riddle *et al.* 2016). These promising studies support the use of O-Ag-based conjugates for human vaccination. Efforts have also been addressed to the synthesis of *Sh. flexneri* 2a and 3a O-Ag through rational investigation of minimal structural epitopes and impact of O-acetylation pattern in the structures (Boutet and Mulard 2008; Vulliez-Le Normand *et al.* 2008; Phalipon *et al.* 2009; Gauthier *et al.* 2014). The conjugate of a synthetic pentadecasaccharide composed of three consecutive repeating units of *Sh. flexneri* 2a O-Ag, developed at the Pasteur Institute in France, has been recently tested in a phase-1 study (van der Put *et al.* 2016).

Vibrio cholerae Cholera is a severe dehydrating diarrheal disease caused by toxigenic strains of Gram-negative *V. cholerae*. It represents a major international health concern: ∼3–5 million cases of cholera and 100 000–130 000 deaths due to cholera occur each year globally (WHO 2010). Children, especially younger than 5 years of age, are at particular risk in endemic areas.

There are more than 200 serogroups of *V. cholerae*, classified based on the O-Ag specificity. Among these serogroups, cholera is mainly caused by *V. cholerae* serogroup O1 and less commonly by serogroup O139. O1 can be classified into two serotypes, Ogawa and Inaba, whose O-Ags are linear homopolymers of α-(1→2)-linked 3-deoxy-*glycero*-tetronamido-D-perosamine, differing for the non-reducing end terminal perosamine unit that is 2-O-methylated only in the Ogawa serotype (Table 2) (Chatterjee and Chaudhuri 2003).

Killed oral cholera vaccines are increasingly becoming a standard cholera prevention and control tool. However, their use has been hampered by the requirement of two or three priming doses, relatively short-term protection and responses of lower magnitude and shorter duration in young children (Balakrishnan 2017).

Conjugates from detoxified Inaba LPS linked to cholera toxin (CT) variants CT-1 and CT-2 were shown safe in adult volunteers and induced anti-LPS vibrocidal antibodies (Gupta *et al.* 1998). Recently, O1 Ogawa and Inaba O-Ags conjugated to recombinant tetanus toxoid heavy chain fragment (TThc) induced carbohydrate specific immune responses and resulted respectively in 95% and 55% protective efficacy in a mouse survival cholera challenge model (Alam *et al.* 2014; Sayeed *et al.* 2015). Likewise,

O139 linked to TT induced specific antibodies that were vibriocidal and protective in the neonatal mouse model of cholera infection (Boutonnier *et al.* 2001). To avoid LPS toxicity, synthetic oligosaccharides deriving from O1 and O139 O-Ag have also been produced for conjugation to carrier proteins (Saksena *et al.* 2006; Soliman and Kovac 2016). While a Ogawa type hexsaccharide bound to BSA induced antibodies that were protective in a challenge assay (Chernyak *et al.* 2002; Rollenhagen *et al.* 2009), the Inaba counterpart linked to *r*EPA induced specific antibodies which were neither vibriocidal nor protective in the infant mouse cholera model (Wade *et al.* 2006). The elicited antibodies, regardless of serotype, were cross-reactive to heterologous LPS; however, anti-Ogawa serum did not kill Inaba bacteria, suggesting that immunodominant LPS epitopes differ between Ogawa and Inaba LPS.

Other surface carbohydrates

In addition to CPS or O-Ag, other carbohydrates decorating the surface of microbial pathogens have been targeted for the development of glycoconjugate vaccines.

A typical example is given by Gram-positive Group A *Streptococcus* (GAS), which is a major cause of pharyngitis in children across the world, with a high frequency of severe sequelae in low- and middle-income countries including acute rheumatic fever, rheumatic heart disease and post streptococcal glomerulo nephritis (Mitchell 2003). Native GAS capsule has the same structure as mammalian hyaluronic acid; thus, it is not a suitable target for a polysaccharide-based vaccine (MacLennan 1956). In contrast, the Lancefield group A carbohydrate (GAC), comprising a polyrhamnose backbone with an immunodominant GlcNAc side chain (Table 2), is a virulence factor, and conjugated to TT carrier protein elicited a protective immune response against systemic or nasal challenge with GAS (Sabharwal *et al.* 2006). An inverse relationship between high Ab titers against GAS PS and the presence of GAS in the throat of Mexican children was observed (Sabharwal *et al.* 2006). The epitope recognized by human antisera was identified in the hexamer composed by two repeating units (Michon *et al.* 2005). Based on this observation, synthetic GAS oligosaccharides (2 or 4 repeats) conjugated to CRM$_{197}$ were demonstrated as efficacious as the conjugated PS in protecting mice from challenge with the pathogen (Kabanova *et al.* 2010). Concerns about GlcNAc-containing vaccines have been raised due to a possible role of anti-GAS-PS antibodies in the development of GAS infection sequelae, like acute rheumatic fever or Sydenham's chorea (Shikhman, Greenspan and Cunningham 1993; Malkiel *et al.* 2000; Kirvan *et al.* 2006). GlcNAc-deficient GAS PS conjugated to recombinant pneumococcal protein SP0435 was also able to induce Abs promoting opsonophagocytic killing of multiple GAS serotypes and able to protect against GAS challenge after passive immunization (van Sorge *et al.* 2014).

Polyrhamnan, besides being a component of GAS PS backbone, is constantly expressed as cell wall polysaccharide in *St. pyogenes* and *St. agalactiae* (van Sorge *et al.* 2014).

The ubiquitous exo-polysaccharide PNAG (Table 2) appears to play an important role in biofilm formation, immune evasion and pathogenesis in a variety of bacterial species including *S. aureus* (Cerca *et al.* 2007), *S. epidermidis* (Mack *et al.* 2004), *Actinobacillus* species (Kaplan and Mulks 2005) and *E. coli* (Wang, Preston and Romeo 2004; Cerca and Jefferson 2008).

When native PNAG from *S. aureus* (90% O-acetylated) was chemically treated to reduce acetylation to 15%, the resulting de-acetylated PNAG glycoform (dPNAG) elicited opsonic and protective antibodies against *S. aureus* (Maira-Litran *et al.* 2002, 2005). In contrast, antibodies specific to the acetylated form were poorly opsonic and not protective (Maira-Litran *et al.* 2004). Notably, most humans (95%) have high titers of natural antibody directed to the acetylated epitopes of native PNAG, and this antibody is poorly opsonic and not protective in animal models (Perez *et al.* 2009). A synthetic dPNAG composed of nine repeating units conjugated to staphylococcal non-toxic mutant of alpha-hemolysin (Hla H35L) induced carbohydrate specific antibodies that reduced the bacterial burdens in animal models of *S. aureus* (skin abscesses, pneumonia and nasal colonization) (Pozzi *et al.* 2012). Carrier-protein specific immunity was also shown to be effective in reducing bacterial levels in infected lungs and in nasal colonization. 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 (Bentancor *et al.* 2012). Immunization significantly reduced post infection levels of *A. baumannii* in the lungs or blood, compared to control groups, demonstrating that the PNAG conjugate could prevent pneumonia and pathogen caused bacteremia.

Another ubiquitous glycan is represented by the enterobacterial common antigen (ECA) (Table 2) (Männel and Mayer 1978). Monomer and dimer of this trisaccharide repeating unit have been synthesized and conjugated to BSA for the development of a specific mAb (SM250–1 A5), which recognized a variety of species, such as *K. pneumoniae*, *Sh. sonnei*, *Sh. flexneri*, *Citrobacter freundii*, *E. coli*, *Y. enterocolitica*, *Enterobacter aerogenes*, *S.* Typhimurium, while it was not reactive to other Gram-negative bacteria (*V. cholerae*) and Gram-positive bacteria (*Listeria monocytogenes*) (Liu *et al.* 2015) .

Alginate, a polysaccharide made by variable ratios of mannuronic to guluronic acids partially O-acetylated, was found in mucoid strains of PA (Pier *et al.* 1994; Pier 2005; Sharma, Krause and Worgall 2011). Conjugated to a variety of carrier proteins, including EPA, TT, KLH, OMV (from *N. meningitidis* serogroup B) or synthetic peptide containing T- and B-cell epitopes, alginate was successful in inducing protective immunity in mice, mediated by opsonophagocityc antibodies at preclinical level, but has never been tested in human (Theilacker *et al.* 2003; Kashef *et al.* 2006; Doring and Pier 2008; Farjah *et al.* 2014, 2015). The structurally similar polymannuronic acid conjugated to type a flagellin also exhibited protective efficacy in a mouse lung infection model (Campodonico *et al.* 2011).

Pel and Psl are other exopolysaccharides produced by PA (Ma *et al.* 2006, 2007; Colvin *et al.* 2012; Jennings *et al.* 2015). Psl is found in 76% of analyzed clinical isolates, and its expression not only occurs in the primary infecting strains, but has also been implicated in establishing a persistent infection. Psl is responsible for the formation and maintenance of biofilms and an anti-Psl mAb exhibited opsonophagocytic killing of a number of strains and conferred significant protection in multiple animal models (DiGiandomenico *et al.* 2012).

TAs have been explored as potential vaccine candidates for Gram-positive bacteria accounting for a large proportion of hospital-acquired antibiotic-resistant infections, such as staphylococci and enterococci (Theilacker *et al.* 2004). Immunization of mice with (poly)glycerolphosphate backbone induced in mice antibodies which mediated opsonophagocytic killing *in vitro* of *S. epidermidis* and *S. aureus* (Table 2) and, upon passive transfer, reduced mortality in a murine *S. aureus* peritonitis model (Theilacker *et al.* 2006). Although LTA has been reported to be an TLR2 agonist, this activity might derive from contaminating lipoproteins/lipopeptides

(Zahringer *et al.* 2008). After conjugation to TT, a synthetic version of the (poly)glycerolphosphate backbone was also able to elicit murine-specific IgG enhancing opsonophagocytic killing of live *S. aureus in vitro*. Mice actively immunized with the (poly)glycerolphosphate conjugate vaccine showed a rapid clearance of staphylococcal bacteremia *in vivo* and, in contrast to purified LTA, did not exhibit detectable inflammatory activity (Chen *et al.* 2013).

LTA substituted or not with alanine have been isolated and recognized by opsonic antibodies against enterococci (Theilacker *et al.* 2006; Sava *et al.* 2010). The kojibiose–TA structure of one type of LTA (Table 2) isolated from *En. faecalis* strains was very similar to the CPS (Wang *et al.* 1999). To circumvent the polymer heterogeneity, various syntheses for the preparation of defined LTA antigens have been described, including automated solid-phase synthesis (Hogendorf *et al.* 2010, 2011, 2012).

A short LTA hexamer with a single disaccharide substituent conjugated to BSA elicited the production of antibodies in rabbit that induced opsonic killing of enterococci and *S. aureus* strain MW2 (Laverde *et al.* 2014). The LTA from *E. faecalis* strain 12030 was found identical to the CPS, except for the substitution at position C-2 with D-alanine (Theilacker *et al.* 2006). Opsonic serum recognized this polysaccharide similarly to LTA lacking of alanine (Sava *et al.* 2010), indicating that anti-LTA antibodies are cross reactive irrespective of the different polysaccharide decorations.

In addition to TA, other classes of heteroglycans have been isolated from enterococci, including a heteroglycan composed of rhamnose, glucose, galactose, mannosamine and glucosamine (Hsu *et al.* 2006). Two polysaccharides containing altruronic acid and legionaminic acid, respectively, and the fructose homopolymer levan have been isolated from *En. faecium*, in addition to a glucosylated LTA (Kodali *et al.* 2015). Immunization of rabbits with CRM$_{197}$ conjugates of these polysaccharides showed that while antibodies raised against levan failed to mediate opsonophagocytic killing, the other two conjugated polysaccharides elicited antibodies with opsonic activity, which were also capable of reducing bacterial load in mouse liver or kidney tissue. Fragments of the capsule-like diheteroglycan (DHG) (Table 2) were recently synthesized and characterized as a promising vaccine candidate (Krylov *et al.* 2015).

Clostridium difficile is a Gram-positive anaerobic spore-forming bacterium, the incidence and severity of which infection appears increased in the last decades, particularly in the USA and Canada (Warny *et al.* 2005). An hypervirulent strain (NAP1/027/BI) with particularly severe antibiotic resistance was identified as the cause of these outbreaks.

Three surface polysaccharide structures, PSI, PSII and PSIII (Table 2), have been isolated (Ganeshapillai *et al.* 2008). PSII was found the more abundant in the hypervirulent strain NAP1/027/BI and many other clinical isolates (Danieli *et al.* 2011; Oberli *et al.* 2011). Conjugates of the polysaccharide or the synthetic repeating unit with CRM$_{197}$, recombinant bacterial toxins or ETEC proteins elicited carbohydrate specific antibodies (Adamo *et al.* 2012; Bertolo *et al.* 2012; Romano *et al.* 2014). By microarray screening of synthetic structures, higher levels of IgA antibodies against PSI were found in patients with low or high severity of disease compared to asymptomatic control (Martin, Weishaupt and Seeberger 2011; Martin *et al.* 2013b). Synthetic glycans selected by epitope mapping studies and displayed on a synthetic scaffold were shown to be immunogenic as larger fragment, demonstrating the candidacy of small structures for vaccine development (Broecker *et al.* 2016a). PSIII is an LTA-like polymer, and anti-PSIII antibodies have also been detected in the

blood of infected patients (Martin *et al.* 2013a). Immunizations with conjugates of intact or de-O-acylated PSIII fractions raised IgG antibodies recognizing the polymer on *C. difficile* cellular surface (Cox *et al.* 2013).

Serum, but not fecal, anti-PSIII IgG were found in hospitalized individuals, possibly due to pre-exposure to the pathogen (Broecker *et al.* 2016b). A synthetic LTA fragment conjugated to CRM$_{197}$ elicited in mice antibodies that bound to the surface of a series of *C. difficile* strains. The alum-adjuvanted CRM$_{197}$ conjugate reduced the bacterial colonization in mice challenged with live *C. difficile* cells.

Mycobacterial and fungal surface carbohydrates

Although an anti-tuberculosis vaccine (BCG) is available, the overall effective rate of prevention is inferior to 50%. The emergence of antibiotic-resistant strains, particularly in HIV-infected individuals, is highlighting the need of improvement of the current preventive therapy. Lipoarabinomannan (LAM, Table 2) is a major lipoglycan component of the outer cell wall of all mycobacterial species. LAM is an important immunomodulating compound, contributing to the pathogenesis of mycobacterial tuberculosis (MBT) infections. The derived AM, obtained by removal of the lipid in order to avoid any immunosuppressive effect, was covalently linked to the mycobacterial protein Ag85B or TT showing protective efficacy in mice and guinea pigs in terms of prolonged survival and reduced pathology, when administered with L3 adjuvant using a subcutaneous priming-intranasal boost regime (Hamasur *et al.* 2003; Kallenius, Pawlowski and Hamasur 2008). Attempts to use conjugates of synthetic mannans (Leelayuwapan *et al.* 2017), phosphatidylinositol mannosides (Boonyarattanakalin *et al.* 2008) or rhamnans (Vignal *et al.* 2003; Meng *et al.* 2017) are in progress, although cross reactivity against MBT has not been so far elucidated.

Candida species have become the fourth most common nosocomial bloodstream isolate in the USA and in most European countries, and a vaccine would be beneficial particularly for patients at high risk like those in intensive care units (Cutler *et al.* 2007; Cassone 2008). Resistance to therapeutic treatment is increasing among *Candida* species and particular concern is being raised by the emergence of *Candida auris* in health care settings because this fungus is very resistant to drugs and can be easily spread from person to person. Branched β-(1→3)-(1→6)-glucans have been considered to develop vaccines against infections caused by *Candida* species. β-Glucans are component of most, if not all, fungi and therefore are attractive targets for fungal vaccines development. Conjugates based on β-glucans and mannans (Table 2), either extracted from a natural source or chemically synthesized, have been proposed as possible vaccine candidates (Torosantucci *et al.* 2005; Bromuro *et al.* 2010; Adamo *et al.* 2011, 2014; Hu *et al.* 2013; Johnson and Bundle 2013; Lipinski *et al.* 2013; Paulovicova *et al.* 2013; Liao *et al.* 2015, 2016). β-Glucans are immunostimulator molecules through Dectin-1 activation (Donadei *et al.* 2015); thus, bicomponent β-glucan and β-mannan conjugate has also been made (Lipinski *et al.* 2013), showing enhanced immunoresponse against the latter antigen.

More recently, other species are emerging as targets for vaccination, such as *Cr. neoformans* and *Aspergillus*. *Cryptococcus neoformans* is an opportunistic encapsulated yeast that causes cryptococcal meningoencephalitis (cryptococcosis) in immunocompromised individuals, including AIDS patients and organ transplant recipients (Cogliati 2013). Invasive aspergillosis (IA), caused by *Aspergillus*, is the second most common cause of nosocomial, invasive fungal infections, with an incidence of

approximately 5 per 100 000 population in the USA (Wilson *et al.* 2002).

Similarly to *Candida*, these species are surrounded by β-(1→3)-glucans (Maubon *et al.* 2006), and capacity of anti β-(1→3)-glucan antibodies to inhibit the cellular growth of acapsular *Cryptococcus* strains has been proven (Rachini *et al.* 2007).

Both *Cryptococcus* and *Aspergilli* present at their surface α-(1→3)-glucans (Table 2). In the first case, they anchor the polysaccharide capsule to the cell wall. In *Aspergillus fumigatus* (Fontaine *et al.* 2010), α-(1→3)-glucans induce the aggregation of germinating fungal conidia. Antibodies elicited in mice by conjugated synthetic α-glucans recognize the cell wall of *Aspergillus* (Komarova *et al.* 2015).

Cryptococcus neoformans also exhibits a peculiar CPS. Four serotypes, A, B, C and D (Table 2), are distinguished and they are composed for a large extent of mannose trimers with glucopyranosyluronate and xylopyranosyl substituents (GXM) creating the so called 'triads' (Cherniak, Jones and Reiss 1988). Strains of serotype A and D are the most frequent cause of cryptococcosis in humans and thus the serotypes of primary interest for a human vaccine (Cherniak and Sundstrom 1994).

A glycoconjugate vaccine composed of unfractionated GXM polysaccharide conjugated to bovine gamma globulin was initially seen highly immunogenic, but protection from infection was not achieved (Goren and Middlebrook 1967). Fractionated GXM polysaccharide conjugated to TT was again highly immunogenic (Devi *et al.* 1991), and both active and passive immunization of mice conferred protection against experimental cryptococcosis (Casadevall *et al.* 1992; Devi 1996). However, using a library of mAbs it was later observed that the GXM-TT vaccine elicit protective, non-protective and even deleterious (disease-enhancing) antibodies (Mukherjee, Scharff and Casadevall 1992; Mukherjee *et al.* 1995). The free unconjugated GXM polysaccharide would also have potent immunosuppressive properties (Vecchiarelli 2000).

These early findings led to the hypothesis that conjugated GXM could contain protective and non-protective epitopes, and a non-protective antibody response would prevent the action of formed protective antibodies (Nakouzi *et al.* 2009). Efforts are currently ongoing to identify the protective and the non-protective epitopes present in the GXM through a library of synthetic oligosaccharides followed by conjugation to protein carrier and testing in mice (Oscarson *et al.* 2005; Guazzelli, Ulc and Oscarson 2015; Guazzelli *et al.* 2015; Guazzelli, McCabe and Oscarson 2016).

CONCLUSIONS

Glycans coating the bacterial surface are key antigens for the development of vaccines against bacterial and fungal diseases. Over the last decades, glycoconjugate vaccines licensed to combat *N. meningitidis*, *H. influenzae* and *St. pneumoniae* have proven to be safe, efficacious and cost effective (Sharma *et al.* 2012; Delgleize *et al.* 2016; Zarei, Almehdar and Redwan 2016; Delea *et al.* 2017; Linares-Perez *et al.* 2017).

Nevertheless, the continuously evolving epidemiology requires additional efforts to extend the coverage, as in the case of *St. pneumoniae*, while other pathogens request to be incessantly monitored to be prepared in case inclusion of emerging serotypes in the vaccine composition would be needed (i.e. *N. meningitidis* serogroup X or *H. influenzae* type a) (Micoli *et al.* 2013b; Cox *et al.* 2017; LaForce 2017b)

Many diseases still remain to be controlled. Over the last years, alarming concern is emerging toward antibiotic-resistant bacteria, including, among others, the so-called ES-KAPE pathogens, which are leading causes of nosocomial infections through the world (Boucher *et al.* 2009; Garcia-Quintanilla *et al.* 2016; Bloom, Black and Rappuoli 2017). Fungal infections are also becoming increasingly invasive for immunocompromised patients, such those with immune systems impaired by cancer chemotherapy, or hospitalized individuals (Spellberg 2011). Recent analyses have highlighted the need of novel therapeutic approaches to tackle these pathogens, and WHO and CDC have ranked and prioritized the relevance of these bacterial targets (CDC 2013; WHO 2017). Other emergencies could derive from bacteria which spread during environmental calamities (*V. cholerae*), particularly impacting poor countries, or which could be used as bioterrorism weapons (*B. pseudomallei* and *mallei*, *F. tularensis*).

Additional factors which will increase the need of preventative therapies are the changes in the age population composition, shifting toward a larger presence of elderly as consequence of the increased life expectation in the developed countries; the increased numbers of travelers, as well as the migration flows from the south to the north of the world; and even climatic changes which could play a role in selecting dangerous pathogens (Rappuoli *et al.* 2011).

Nowadays different tools are available for the manufacturing of glycoconjugates, which could accelerate the development of novel carbohydrate-based vaccines. Semisynthetic conjugation is a well-established approach which delivered to patients efficacious vaccines against deadly pathogens. It requires a complex multistep manufacturing and quality control flow; however, it will continue to represent a reference approach, particularly for those structures difficult to express by glycoengineering methods and not promptly accessible by chemical synthesis.

The synthetic approach has been considered time demanding and expensive for vaccine manufacture compared to the use of bacterial-derived carbohydrates. Nonetheless, clear proofs of feasibility have been provided by the marketed synthetic Hib vaccine (Verez-Bencomo *et al.* 2004) and the pentadecasaccharide *Shigella* conjugate recently entered phase-1 clinical trial (van der Put *et al.* 2016). Additionally, synthesis of a number of challenging pneumococcal serotypes conjugates has been recently achieved (Geissner *et al.* 2016; Parameswarappa *et al.* 2016; Emmadi *et al.* 2017; Lisboa *et al.* 2017; Schumann *et al.* 2017).

Synthetic carbohydrate chemistry can aid identification of the sugar target epitope, optimal carbohydrate density and attachment site to the carrier protein, supporting the rational design of vaccines with improved immunogenicity and preserved key protective protein epitopes.

Bioconjugation will play a pivotal role for faster and cheaper production of multicomponent vaccines. Bioconjugation has already provided candidates against *Sh. dysenteriae* O1 and *Sh. flexneri* 2a (Hatz *et al.* 2015), and against ExPEC (Huttner *et al.* 2017) tested in phase-1 clinical studies. *Escherichia coli* N-glycosylation so far applied is limited to the use of PglB for the transfer of the nascent polysaccharide chain with an end-terminal NAc hexosamine to the carrier protein. Directed enzyme evolution is applicable to increase the transferase promiscuity toward other sugar acceptors (Ihssen *et al.* 2015). PglL, the O-transferase from *N. meningitidis* (Musumeci *et al.* 2013), and NCT from *Ac. pleuropneumoniae* (Cuccui *et al.* 2017) could also give access to a broader range of hexose ending polysaccharides.

Examples so far achieved of bioconjugate vaccines are related to polysaccharides assembled through group 1 and 4 CPS biochemical pathways. Other groups are more challenging and represent an opportunity to further progress this technology.

The use of glycoengineered OMVs appears as a flexible but at a very early stage approach. Synthetic oligosaccharides and bioconjugation will aid the development of vaccines with higher standards in terms of product manufacturing and characterization compared to traditional conjugate vaccines.

The present analysis underscores how carbohydrate antigens will be key for the development of future vaccines against emerging pathogens, primarily the ESKAPE bacteria *A. baumannii, En. faecium, K. pneumoniae, P. aeruginosa*, classified and critical targets by WHO and CDC, as well as *C. difficile* and GAS, categorized as serious threats. Vaccination for prevention of relevant targets, such as *S. aureus* and GBS infections, is progressing at clinical level. Fungal infections (*Ca. albicans, Cr. neoformans*) could also benefit of carbohydrate-based vaccination. Among the pathogens discussed in this review there are examples where a simple vaccine formulation with one or two antigens has the potential to ensure large coverage (e.g. *F. tularensis, B. pseudomallei* and *mallei, V. cholerae, Haemophilus* type a), and the development of a glycoconjugate vaccine appears feasible. For *M. catarrhalis, N. gonorrhoeae* and NTHi LOS, alone or in combination with protein antigens, could be key targets for vaccine design. In most of the other cases, a combination of multiple sugar components would be required. This is the case of *Klebsiella*, for which O-Ag could ensure a better coverage with a smaller number of antigens as compared to the CPS, or *E. coli*. In other cases, such as PA, the complexity of the bacterial mechanism of infection with transition from acute to chronic phase might render more challenging the identification of the optimal antigens. Use of pathogen-derived proteins as carrier for the glycan haptens could reduce the number of vaccine components, by broadening across strain protection and/or by tackling the pathogen on different virulence factors, provided that the key protective epitopes of the protein carrier are identified and preserved during conjugation (Broker *et al*. 2017).

Recent advances in the field of glycoconjugate vaccines, synergy of the different technologies currently available and their appropriate selection will enable the tailored design of glycoconjugates of novel targets, expanding the number of available vaccines and tackling currently unmet medical needs.

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