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Serum antibody screening using glycan arrays

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Humans and other animals produce a diverse collection of antibodies, many of which bind to carbohydrate chains, referred to as glycans. These anti-glycan antibodies are a critical part of our immune systems' defenses. Whether induced by vaccination or natural exposure to a pathogen, anti-glycan antibodies can provide protection against infections and cancers. Alternatively, when an immune response goes awry, antibodies that recognize self-glycans can mediate autoimmune diseases. In any case, serum anti-glycan antibodies provide a rich source of information about a patient's overall health, vaccination history, and disease status. Glycan microarrays provide a high-throughput platform to rapidly interrogate serum anti-glycan antibodies and identify new biomarkers for a variety of conditions. In addition, glycan microarrays enable detailed analysis of the immune system's response to vaccines and other treatments. Herein we review applications of glycan microarray technology for serum anti-glycan antibody profiling.

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1. Introduction

Antibodies are immunoglobulin proteins produced by the immune system as part of the host defense system. Their key function is to bind target molecules, referred to as antigens, with high affinity and specificity. Binding of antibodies to antigens displayed by a microbe initiates a variety of outcomes, including direct neutralization *via* blocking an essential process, induction of killing *via* the complement pathway, or tagging for elimination by cells of the immune system. Binding to toxins or other macromolecules can block their function and mediate their clearance from the body. While these antibody-mediated processes are usually beneficial for host defenses, they can become detrimental in the absence of proper regulation. For example, the production of antibodies that recognize self-antigens can cause autoimmune diseases.

Mammals produce a complex and diverse repertoire of antibodies. The specificity of each antibody varies based on its sequence, with different antibodies targeting different antigens. The number of unique antibodies in a human is

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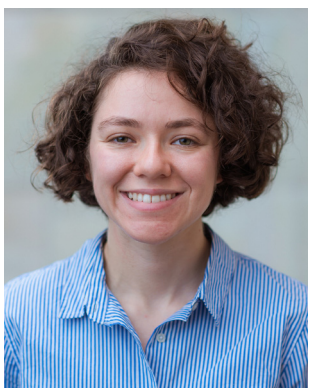

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estimated to be in the billions, and these serum antibodies collectively recognize an amazingly vast assortment of antigens, including proteins, lipids, nucleic acids, and carbohydrates. Serum antibody repertoires have been extensively studied by B cell receptor sequencing studies,[1] antibody mass spectrometry immunoproteomics,[2] protein and peptide microarrays,[3,4] and glycan microarrays.

The antibody repertoire of each individual is unique and determined by a variety of factors. Genetics and random chance both influence which antibody V, D, and J genes are initially joined to produce full-length antibodies, and diversity can be further increased by somatic recombination and hypermutation.[5] Environmental circumstances—such as vaccination, disease history, and exposure to various microorganisms, allergens, and toxins—also play a key role. Because these factors vary over time, the antibody repertoire changes throughout life.

Given that antibodies are both critical for immune protection and reflective of an individual's medical and genetic history, a person's endogenous antibody profile provides vital information about their health. Serum antibodies are already used extensively as diagnostic markers for various diseases, such as HIV infection, Lyme disease, and mononucleosis. Therefore, technologies that enable rapid and comprehensive analysis of serum antibodies have further applications to diagnostics, treatments, and many other aspects of medical research and care.

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Prof. Vered Padler-Karavani received BSc in biology, MSc and PhD in biochemistry, all from Tel Aviv University, and postdoctoral training with Prof. Ajit Varki at The University of California, San Diego. She established The Laboratory for Glycoimmunology at Tel Aviv University to study mechanisms that govern glycan immune recognition and responses, through basic science to translational studies in humans, aiming to develop novel diagnostics and therapeutics to chronic inflammation-mediated diseases. This multidisciplinary research combines glycobiology, immunology, bio-nanotechnology, bioinformatics, statistics, medicine, xenotransplantation and cancer research, and involves cutting edge technologies within these disciplines, including glyco-nanotechnology and glycan microarrays.

1.1. Composition of serum and types of serum antibodies

Blood contains red blood cells, white blood cells, and platelets in a protein-rich fluid. Serum/plasma are the cell-free liquid phase of clotted/un-clotted whole blood, respectively. These solutions contain 60–80 mg ml^{-1} proteins, 40% of which are circulating antibodies (immunoglobulins) that recognize a plethora of diverse antigens. There are five immunoglobulin (Ig) isotypes: IgG, IgA, IgM, IgD and IgE. In normal human serum, IgG is the most abundant (8–20 mg ml^{-1}; ~75% of antibodies), followed by IgA (~1–4 mg ml^{-1}), IgM (~0.5–2 mg ml^{-1}), IgD (~0–0.4 mg ml^{-1}), and trace IgE (<0.00015 mg ml^{-1}).[6,7] IgM is the primary responder to an initial immune stimulus, mediating complement activation and agglutination. Although the interaction between an individual IgM binding pocket and an antigen is generally fairly weak, IgM assembles into pentamers with 10 antigen binding sites, compensating for lower affinity with higher avidity.[8] Class-switching of the B cell can produce IgG, which is divided into four subclasses (IgG1, IgG2, IgG3, and IgG4) that play leading roles in many key

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immune processes, including complex formation, phagocytosis, and fetal/infant passive immunity.[8,9] IgA is most abundant in mucosal secretions of respiratory, gastrointestinal, and urogenital tracts and provides the primary defense against inhaled and ingested pathogens. IgA is largely monomeric in serum and dimeric in secretions.[8,10] IgE is associated with hypersensitivity, allergy, parasitic infection, and fungal infection.[8,11] Though little is known about IgD, its role in serum seems to be related to mucosal immunity, while membrane-bound IgD is implicated in development and regulation of peripheral B cells.[12,13] These different antibody isotypes diversify the effector functions and capacities of antibodies as a whole.

1.2. Glycans as target antigens

Glycans are one of the most abundant biopolymers on the planet (Fig. 1). They coat the surfaces of all cells and many viruses as well. As such, they are readily accessible to the immune system.

Glycomes, the collection of glycans produced by an organism, are dynamic and variable between organisms. The mammalian cell glycome is complex, with many glycans attached to proteins or lipids. On proteins, glycans can be attached to the side chain nitrogen of asparagine to produce *N*-linked glycans (Fig. 1b) or to the side chain oxygen of serine, threonine, tyrosine, or hydroxyproline to produce *O*-linked glycans (Fig. 1c). On lipids, glycans can be attached to form the glycosphingolipids (GSLs) that comprise a major component of mammalian cell membranes (Fig. 1d). Glycosaminoglycans are large polysaccharides that can be attached to proteins to produce proteoglycans or can be found free in the extracellular matrix. *O*-GlcNAcylation (modification with an *O*-linked *N*-acetyl-glucosamine) is another type of glycosylation found on cytoplasmic and nuclear proteins.[14] It is a dynamic post-translational modification analogous to phosphorylation.

Glycans play key roles in biology. In mammalian cells, glycans are involved in processes such as protein trafficking, signaling, protein folding, and cell–cell adhesion. Glycan expression patterns change significantly during disease and can directly mediate disease processes. In microbial cells—like bacteria, fungi, and parasites—polysaccharides are essential for maintaining the integrity of the cell walls and often contribute to pathogenicity in various ways, such as by suppressing the immune response or by promoting biofilm formation. For example, polysaccharides such as chitin and beta-glucans are major components of fungal cell walls, and lipopolysaccharides and peptidoglycans are major components of bacterial cell walls. Microbial glycans have unique structures and often include monosaccharides not found in mammals, making them good target antigens for the immune system.[15]

1.3. Endogenous anti-glycan antibodies

Humans and other animals produce a complex and diverse assortment of antibodies that recognize glycans. Among the first well-characterized antibody responses to carbohydrates were antibodies to the ABH blood group antigens that define

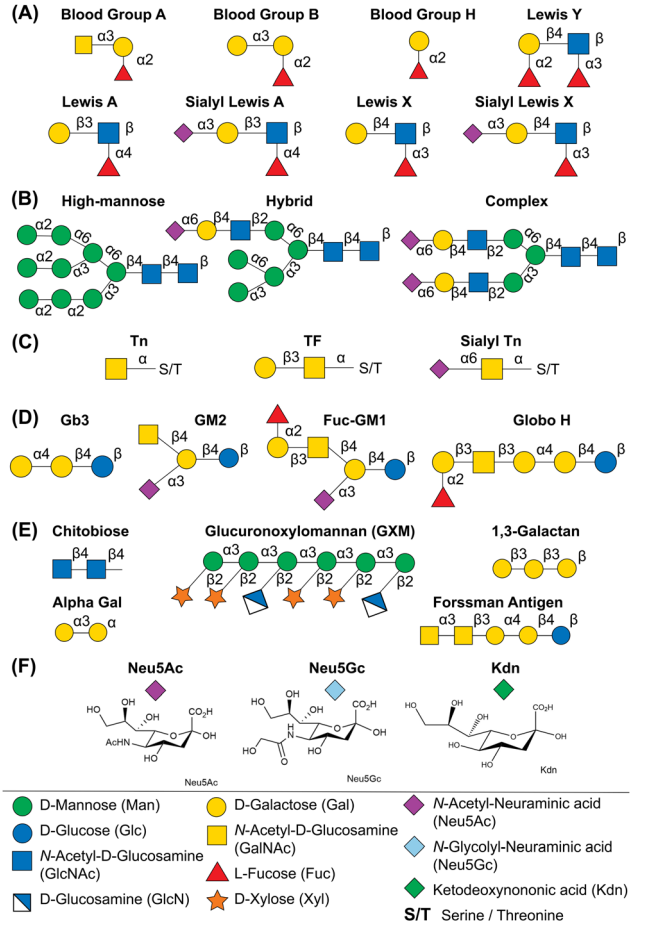

Fig. 1 Representative glycan epitopes for serum anti-glycan antibodies. (A) ABO blood group and Lewis antigens. (B) *N*-linked glycan antigens. (C) *O*-linked glycan antigens. (D) Glycolipid glycans. (E) Bacterial, fungal, and other microbial or non-human glycans. (F) Sialic acids. Note that some structures may fit into multiple groups (*e.g.*, glycolipid and non-human glycan). Glycoglyph was used to produce glycan structures.[16]

ABO blood types.[17] It was determined that individuals produce high titers of antibodies against any ABO(H) blood group antigens not present on their own cells. While the exact source of these antibodies has remained controversial for decades, recent studies have provided evidence to support the hypothesis that they arise from infection or exposure to gastrointestinal bacteria.[18,19] Regardless of the origins of these antibodies, they are clinically important in blood transfusion medicine. If a patient receives a blood transfusion from an incompatible blood donor, these antibodies will bind to blood group antigens on the donor red blood cells, triggering complement fixation and cell lysis. The resulting accumulation of large amounts of cell debris can then cause acute renal failure. The final identification of the ABO(H) antigenic structures in the 1960s—led by Winnifred Watkins, Walter Morgan, Elvin Kabat and others—was achieved before the age of the microarray.[20] Since then, modern studies on antibodies to blood group antigens have been tremendously enhanced by the glycan microarray.[21]

Other early studies on anti-glycan antibodies focused on antibodies generated in response to bacterial infections. The work in the early 1900s through the 1960s, which predates microarray technologies, identified many antibodies to microbial glycans, some of which are present at very high concentrations in serum. For example, serum anti-α-Gal antibodies have been reported to account for as much as 1% of all IgG in humans.[22] Other abundant antibody populations against microbial glycans include those targeting rhamnose, Forssman antigen, and beta-glucans.

While most endogenous anti-glycan antibodies are induced in response to antigen exposure, they can also be part of the "natural antibody" repertoire.[23,24] Natural antibodies are produced by B1 cells in the absence of antigen exposure. In sharp contrast to typical antigen-induced antibodies, most natural antibodies contain few or no mutations from germline variable region sequences, display polyreactive binding with weak affinity, and are maintained at consistent levels over a person's life. Natural antibodies often bind self-glycans and other self-antigens but do not cause autoimmune diseases. Instead, they are believed to be involved in homeostasis and removal of cell debris.[25]

Beyond the set of antibodies naturally produced by the immune system, anti-glycan antibodies can also be induced by vaccination. Numerous carbohydrate-based vaccines are in development, and several have been FDA approved. Some examples include Prevnar 13 and Pneumovax 23 for *Streptococcus pneumoniae* infections, Menactra for *Neisseria meningitidis* infections, and PedvaxHIB for *Haemophilus influenza* type b infections. These vaccines provide protection by inducing antibodies against bacterial polysaccharides. Carbohydrate-based vaccines are also being developed for numerous other applications, such as preventing HIV infection and preventing or treating cancer.[26]

Not all anti-glycan antibodies in serum are beneficial. Antibodies that recognize non-neutralizing antigens or epitopes on a pathogen can divert resources away from more productive responses. In some cases, recognition of the wrong antigen molecules or the wrong epitopes can even perpetuate infection, a phenomenon known as antibody-dependent enhancement.[27] Furthermore, antibodies that recognize self-glycans can lead to autoimmune diseases. For example, antibodies that target the gangliosides GM1a and GD1a are found in patients with Guillain–Barré syndrome and are associated with neurological problems.[28] Another type of serum anti-glycan antibody, cold agglutinins, can mediate an autoimmune condition called cold agglutinin disease. In healthy people, cold agglutinins only bind self-glycans at low temperatures (typically ~ 4 °C, but anything < 30 °C), but in cold agglutinin disease they acquire the ability to bind at warmer temperatures, allowing them to recognize self-glycans and cause haemolysis and other problems, especially in a patient's extremities.[29]

Serum anti-glycan antibodies are appealing to study in large part due to their easy accessibility: they are present in blood. Moreover, serum antibodies are often induced at an early stage of disease and can amplify a small amount of tumor or other antigen into a large biomarker signal. Together, these characteristics make serum antibodies a promising tool for early diagnosis of cancer, infectious disease, autoimmunity, and other pathologies. Using serum antibodies as diagnostic biomarkers could also improve clinical sensitivity and specificity by complementing existing tests or providing cheaper, faster, or more convenient alternatives.

1.4. Glycan microarrays for serum profiling

Traditionally, profiling serum anti-glycan antibodies has been laborious. One could only evaluate antibodies to a few carbohydrate antigens at a time, and the assays required substantial time and materials. As a result, anti-glycan antibodies have been significantly under studied relative to other populations of antibodies, such as those against protein antigens.

Glycan microarray technology resolves many of these barriers. Glycan microarrays contain many different glycans and glycoconjugates, including glycopeptides, glycoproteins, glycolipids, or unnatural glycoconjugates. These array components can be immobilized on a solid support, displayed on phage particles, suspended in liquid format, or barcoded with DNA. Glycan microarrays provide a high-throughput platform to simultaneously evaluate many different anti-glycan antibody subpopulations in parallel. As such, they are especially useful for antibody discovery efforts, where one does not know *a priori* which subpopulations are relevant to the disease or condition. With other technologies, analyses may be biased towards candidate antibodies that are anticipated to be relevant, potentially overlooking many strong binders. Simultaneous evaluation of many glycan-binding antibody subpopulations additionally enables users to identify sets of complementary antibodies that work in tandem. Glycan microarray assays typically only require a few microliters of serum or plasma, posing a relatively small burden on precious and limited supplies of banked samples.

Herein, we review applications of glycan microarrays for profiling serum anti-glycan antibodies. This technology has been used extensively to understand and characterize the immune repertoires of healthy subjects, as well as to study responses to infectious disease, cancer, autoimmune disease, and transplants. Glycan microarrays have also proven valuable for evaluating antibody responses to vaccination and identifying novel glycan targets in diseased states.

2. Glycan array platforms and fabrication

The ability of antibodies to bind glycans can be assessed *via* many different technologies, collectively referred to as "display technologies", which include thin-layer chromatography,[30–32] ELISA-type formats,[33,34] and carbohydrate libraries encoded on beads.[35] A more modern, high-throughput approach to assess antibody binding is the printed glycan microarray. The term "microarray" references both the micro-quantities of glycans required for array fabrication, as well as the tiny surface area

(typically ~50–200 microns in diameter) in which the glycan is presented. Large, diverse glycan microarrays are powerful tools for antibody discovery, especially when specific target antigens of interest are not yet known. Conversely, smaller glycan microarrays containing variations of a limited number of glycans may be used to optimize the structure of the target antigen or to distinguish between closely related structures. These two formats provide valuable and complementary information applicable to many aspects of serum profiling. A variety of platforms and fabrication methods are available for the design and construction of glycan arrays.[36–38] A brief overview is described below. For more details, see several recent reviews.[39–48]

2.1. Printed glycan arrays

In printed glycan microarrays glycans are immobilized onto slide surfaces in a spatially defined arrangement (Fig. 2).[36,43,44,49–53] To achieve this, various slide chemistries and derivatization approaches have been employed. Most non-covalently printed arrays use nitrocellulose-modified slides as a hydrophobic surface to adsorb large polysaccharides, glycolipids, or glycoproteins.[54] Most covalently printed microarrays use *N*-hydroxysuccinimide-(NHS), epoxide-, or aldehyde-modified slides to react with amines on the glycans or on a linker.[52,55–58] For both classes of printing chemistries, arrays are produced by automated printing of nanoliter quantities of the desired glycan or derivative suspended in buffer.

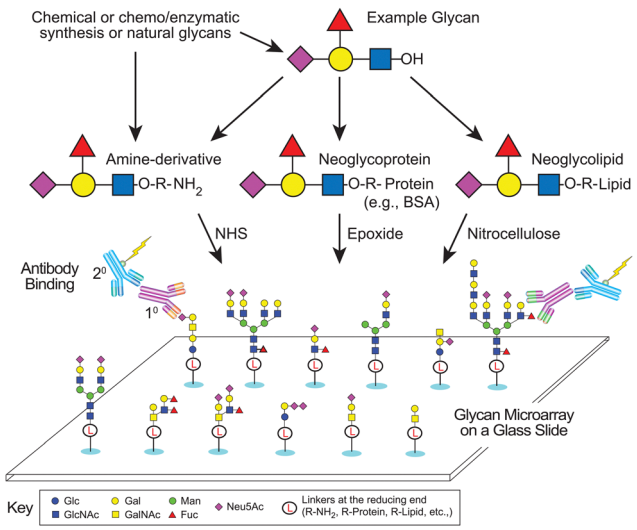

Fig. 2 Preparation of Glycan Microarrays. Glycan microarrays may be generated using free glycans that are either chemo-enzymatically synthesized or derived from nature. Synthetic glycans may have a 'linker' of some type at the reducing end (*e.g.*, aliphatic amine), or they can be derivatized with linkers that have amines. Glycans may also be chemically linked to proteins (neoglycoproteins) or to lipids (neoglycolipids). Glycan-amine derivatives or neoglycoproteins may then be covalently linked to glass slides functionalized with *N*-hydroxysuccinimide (NHS) or epoxide chemistry, and neoglycolipids can be non-covalently linked to slides functionalized with nitrocellulose. Depicted here is a 'defined glycan microarray' in which all glycan structures are known. Because of the difficulty in acquiring many defined glycans, "shotgun glycan microarray" approaches have been developed in which natural glycans are fractionated and then structurally defined after being recognized by an antibody or lectin, as described in the text.

2.1.1. Glycan derivatization. To achieve compatibility with many of the aforementioned glycan microarray formats, glycan antigens may be modified to have a reactive amine at their reducing end. Alternatively, natural/isolated glycans can be modified by either reductive or non-reductive approaches. These may include addition of a bifunctional linker with a terminal amine group[52] or a fluorescent linker, which preserves a reactive amine in the final fluorescently tagged glycan.[59] Linkers provide spacing between the slide surface and flexibility to enable better accessibility and recognition of the glycan. Linkers also offer an opportunity to introduce new functionalities, such as fluorescence, which can be used to detect printed glycans.

For some array platforms, glycans are conjugated to carrier molecules to vary presentation.[60] Glycans can be attached to a protein carrier—most often bovine serum albumin (BSA)—to generate a neoglycoprotein.[56,61] Glycans may also be conjugated to a lipid scaffold to generate a neoglycolipid.[43] Some other examples include glycodendrimers,[62–65] multivalent display on DNA,[66–68] glycoclusters,[69] and glycopolymers.[70–73] Neoglycoproteins, neoglycolipids, and other conjugate formats offer unique advantages for arrays. For example, neoglycoproteins provide glycan spacing resembling that of natural glycoproteins, and these multivalent conjugates can be used in many other experiments, such as ELISAs, Western blots, and as multivalent inhibitors. Neoglycolipids on the array surface have some mobility within the spot, allowing for fluctuations in spacing to match the spacing of binding sites on an antibody, facilitating the formation of multivalent complexes.

2.1.2. Defined *vs.* shotgun/natural arrays. In addition to the "defined glycan microarrays" described thus far, various "non-defined" array formats also exist. Unlike the "defined" format, in which all glycan structures are known prior to printing, "non-defined" arrays include structurally uncharacterized natural glycans, and are often referred to as "natural glycan microarrays"[74–77] or "shotgun glycan microarrays".[78] To generate "non-defined" arrays, total glycans are derivatized, separated by chromatographic means, then printed. After determining which array components are recognized by an antibody or lectin, those specific components can then be revisited for structural analysis and identification. A key advantage of the approach is the ability to evaluate binding to a variety of glycans from tissues/sources of interest without knowing their structures *a priori*. As a result, these arrays have proven highly useful in characterizing antibody responses to unusual glycans from parasites and infectious organisms.[55,79–81] However, obtaining pure compounds and characterizing them can be difficult, and the presence of many uncharacterized glycans should be considered when deciding if this array format is well suited for a particular application.

2.2. Other array formats

Beyond the printed glycan microarray format discussed previously, glycan microarrays have also been generated in the

liquid phase[82] or on beads.[35,79,83] One such bead-based array, the Luminex array, provides a semi-automated platform enabling rapid analysis of hundreds of serum samples against up to 500 glycans per assay.[79,84] A liquid phase array involves multivalent display of glycans on phage particles, with phage DNA encoding the identity of the surface glycan.[82] Other DNA-barcoded glycan array formats have also been developed.[85–87]

3. Profiling normal human serum repertoires

Profiling the repertoire of serum antibodies in healthy subjects enhances our understanding of the immune system and provides a baseline for evaluating immune responses to infection, disease onset and progression, vaccination, and therapy. To identify changes and/or abnormal antibody populations, one must first define normal repertoires. Therefore, understanding healthy repertoires drives the discovery of novel diagnostics and therapeutic approaches.[88–90]

3.1. Serum assay and technical considerations

Early experiments that screened normal human serum on glycan microarrays focused on method development, data processing/analysis, overcoming technical challenges,[91–94] and cross-platform comparisons.[95–97] Many technical factors influence the outcome of a serum profiling array experiment, and consideration of these parameters is important when analyzing results, drawing conclusions, or comparing results from different studies. Several of the most important factors are discussed below.

First, the composition of the array determines what can be measured. Glycan microarrays can only detect antibodies for which there is a suitable glycan antigen present. Currently, the largest glycan microarrays represent only a small fraction of the vast glycan diversity found in nature. Furthermore, the carrier glycan chain or linker displaying the glycan epitope also influences recognition.[98–102] Therefore, there is a need to expand glycan libraries in order to cover more targets. Moreover, beyond the identity of the actual glycans on a microarray, their presentation is also important. For example, different antibody profiles can be detected depending on the density at which the glycan antigens are displayed.[96,103,104] Zhang *et al.* examined 15 sera against a 591-component array consisting of 147 neoglycoproteins printed at varying densities. Dramatically different binding profiles were observed for different neoglycoprotein densities, exemplifying the importance of glycan presentation in distinguishing different IgG subpopulations.[103] For example, some anti-glycan antibodies will only bind when the glycan is present at high density, whereas others will bind at both low and high density.[99] At high density, some antibodies will cross-react with glycans other than the intended target. Thus, the density influences which serum antibodies are captured on a particular array spot. The optimal density is dependent on the goals of the project and typically needs to be determined experimentally. Additional studies to better predict the effects of density would be beneficial.

Second, the assay format and parameters influence the antibody profiles detected on a glycan microarray. Most frequently, serum is diluted between 10- and 200-fold with buffer, then incubated on the array for 2–4 hours at either room temperature or 37 °C. After the primary incubation step, arrays are washed and then incubated with fluorophore-labelled secondary antibodies to detect IgG, IgM, IgA, or all isotypes (anti-Ig). While most early array experiments measured binding of only one antibody isotype or subclass per array, the development of multiplexed microarray approaches has enabled simultaneous detection of IgG, IgA, and IgM on a single array with a single sample.[105] Agents that block non-specific binding may be required throughout these steps. Finally, arrays are washed, dried, and imaged using a fluorescence microarray scanner.

The working dilution, incubation times and temperatures, reagents, buffers, and blocking agents can all affect the antibody binding profile. For example, secondary reagents (*e.g.* Cy3-labeled goat anti-human IgG) are used to detect antibodies bound to the array surface. These reagents must have high specificity for accurate results, so validation is important to ensure reliable data. The dilution also affects the outcome. At low dilutions (high serum concentrations), antibodies at lower abundance can be detected, but those at higher abundance may be saturated (*i.e.*, certain array components have captured the maximum possible amount of antibody). Alternatively, at high dilutions (low serum concentrations), antibodies at lower abundance may be undetectable, but those at high abundance are no longer saturated, so differences in binding strength can be detected. The dilution also affects potential competition between isotypes and/or subclasses. Under low-dilution conditions where antibodies can saturate array components, competition between antibody isotypes may influence the measured profiles. For example, microarrays printed with 412 glycans were examined with pooled human IgG, IgA, or IgM—each in the presence of one other isotype at a time—at increasing concentrations. It was found that IgM outcompete IgG and IgA for many glycan antigens.[106] Interestingly, this result differs for protein-binding antibodies: IgG and IgA outcompete IgM. Competition can be eliminated for most components by carrying out the assay at a higher dilution where most spots are not saturated.

Third, the nature of the sample must be considered. Serum and plasma are different preparations of the fluid part of blood, and they are generally considered interchangeable for antibody profiling.[107] Both serum and plasma can be obtained fresh, but many available samples of interest have been collected and frozen years prior to the experiments. Such samples may undergo several freeze–thaw cycles if they are used in multiple experiments. In our experience, 2–3 freeze–thaw cycles do not affect antibody profiles; however, this may vary depending on the assay parameters. Much less is known about how the collection methods, type of anti-coagulant used for plasma, or sample processing affect the outcome.

Given that many factors can influence the outcome, reproducibility is an important concern. In general, antibody signals measured on arrays have relatively good reproducibility. In one study, for example, replicate serum antibody profiles measured on the same day had an average coefficient of variability (CV) of 10.8%.[108] When evaluated across multiple serum samples, different batches of microarray slides, and different days, the average CV was 29%. Much less is known about the consistency of antibody profiles measured using different glycan array platforms. Several cross-platform comparisons have been carried out using lectins,[57,97,109] but none have been published for serum antibody profiling.

Because all of these technical factors have a substantial effect on the outcome of array experiments, researchers in the community need information about these parameters to properly evaluate the results of a study. Over the last several years, there has been a push to improve the standards for reporting glycan microarray-based data. These guidelines are being established by the MIRAGE (minimum information required for a glycomics experiment) project,[110] and should be considered when designing experiments and examining results.

3.2. Diversity of anti-glycan antibodies in human serum

The repertoire of normal human serum anti-glycan antibodies has been examined extensively on glycan microarrays (Table 1). Wang *et al.* probed one of the first glycan microarrays, containing 48 glycans, with 20 sera.[50] They observed IgM and IgG recognition of diverse microbial polysaccharides, complex cellular carbohydrates, and semisynthetic glycoconjugates. This early study demonstrated the power of microarray technology to explore anti-glycan antibody binding against diverse antigens.[111] In another early study, pooled IgG was profiled on an array with 34 glycans.[112] Blixt *et al.* employed an array of 200 synthetic and natural glycans representative of major glycoprotein and glycolipid structures (*i.e.* ABO, H, LeA, LeB, mannose, α-Gal, gangliosides, peptidoglycan, and rhamnose). Screening 10 sera, they identified combined IgG/A/M against blood group antigens and various human pathogenic bacteria and yeast.[52] Huflejt *et al.* examined 106 serum samples from women against 205 synthetic glycans, detecting combined IgG/A/M against blood group antigens, terminal and core *N*- and *O*-glycans, terminal glycans of glycolipids, tumor-associated carbohydrate antigens, and bacterial/pathogenic polysaccharides and lipopolysaccharides.[113] Bovin and coworkers carried out several screening experiments, including examining 10 sera against 600 synthetic and natural glycans and 15 sera against a 273-glycan array. They identified binding of combined IgG/A/M to over 100 different glycans, including core glycans and antennary polylactosamine,[102,114] and also demonstrated the impact of serum dilution on antibody binding profiles.[115] Follow-up studies examining combined IgG/A/M of 106 sera over 300 glycans yielded similar results.[116] Collectively, these early studies demonstrated that a vast collection of anti-glycan antibodies exists in normal human sera, motivating more detailed exploration of this antibody repertoire.

The glycan-binding antibody repertoire of healthy individuals is defined by the initial set of antibodies present at birth, as well as by additional antibodies developed over time due to

Table 1 Summary of glycan microarrays tested with healthy human serum[a]

Number of glycans	Type of glycans	Number of samples screened	Serum dilution	Glass slide surface	Isotype	Ref.
48	Microbial polysaccharides, complex cellular carbohydrates and semisynthetic glycoconjugates	20	1 : 100	Nitrocellulose	IgM, IgG	50
200	Synthetic and natural glycans	10	1 : 25	NHS	Combined IgG/A/M	52
205	Synthetic glycans	106	1 : 15	NHS	Combined IgG/A/M	113
273	Synthetic glycans	15 blood group O pooled sera	1 : 0, 1 : 15, 1 : 100	NHS	IgG, IgM	115
591	Glycoproteins, neoglycoproteins	15	1 : 50	Epoxide	IgG, IgM multiplex	103
600	CFG v4 synthetic and natural glycans	10	1 : 15	NHS	Combined IgG/A/M	102
45	Neoglycoproteins	30	1 : 50	Epoxide	IgM, IgG	104
128	Glycoproteins, neoglycoproteins	54 (7 + 48)	1 : 50, 1 : 200, 1 : 800	Epoxide	Combined IgG/A/M	108
330	Glycoproteins, neoglycoproteins	135	1 : 50	Epoxide	IgM, IgG multiplex	138
1022	NCFG v1 and CFGv5. 1 synthetic and natural glycans, CFG Microbial Glycan Microarray	105	1 : 50	NHS	IgM, IgG multiplex	111
300	Synthetic glycans	106	1 : 15, 1 : 100	NHS	Combined IgG/A/M	116
31	Neu5Gc-sialoglycans	11	1 : 100	Epoxide	IgG	96
503	Glycoproteins, neoglycoproteins	20 matched maternal-cord blood sets	1 : 5	NHS	IgA, IgM	117
500	Glycoproteins, neoglycoproteins	27 (7 + 20)	1 : 50 to 1 : 200	Epoxide	IgA, IgM, IgG multiplex	105
83	*O*-GalNAc glycans	58	1 : 50	NHS	IgG, IgM multiplex	118
90	*O*-glycopeptides of EBV gp350/220 envelope protein	40	1 : 10	NHS	IgG	119

[a] *N*-Hydroxysuccinimide (NHS); Consortium for Functional Glycomics (CFG); National Center for Functional Glycomics (NCFG); Epstein–Barr virus (EBV).

infection, vaccination, and exposure. One strategy to evaluate the initial set of antibodies present from birth is to compare maternal *versus* fetal serum antibodies. IgG from the mother can cross the placenta during gestation, but IgM cannot. As a result, fetal repertoires are comprised of maternal IgG and fetal IgM. Interestingly, profiling of IgM from umbilical cord blood and matched maternal sera on glycan microarrays revealed that cord IgM from different newborns were highly similar to each other, but distinct from maternal IgM repertoires.[117]

Several glycan microarrays have been designed with a particular focus on *O*-glycans. The first is an extensive *O*-GalNAc (*N*-acetyl-galactosamine) glycan microarray with 83 different mucin-type *O*-glycans conjugated to serine or threonine residues. This comprehensive glycan library was generated by chemoenzymatic modification of *O*-GalNAc cores 1, 2, 3, 4, and 6 to produce diverse natural epitopes including sialylated structures, Lewis and blood group antigens, and Cad/Sd[a] antigens. When applying this array to investigate *O*-glycan recognition by normal human sera IgG and IgM, serum IgG showed binding only to *O*-glycans presenting the A or B blood groups. IgM binding patterns were more variable between individuals, with generally higher binding to cores 3/4/6 than cores 1/2, even though the cores 3/4/6 are much rarer on cells. In addition, IgM bound more strongly to glycans on serine than threonine.[118] Another array was designed by on-slide enzymatic *O*-glycosylation of 20 mer stretches of the Epstein–Barr virus glycoprotein 350/220. Serum screening demonstrated a diverse serum IgG binding pattern to this collection of *O*-glycopeptides.[119]

To explore the characteristics of anti-glycan antibodies at near-population levels, pooled human Ig has been examined (see Table 2). Von Gunten *et al.* profiled pooled human intravenous IgG (IVIG) collected from thousands of healthy individuals from three different commercial sources (Sandoglobulin, Privigen, Gamunex) against 377 glycans.[120] There was great similarity in IgG repertoires of IVIG from different donor populations, with IgG consistently binding ~50% of printed glycans. Strong binding was observed to bacterial glycans such as lipopolysaccharide, lipoteichoic acid, capsular polysaccharides and exopolysaccharides, but not to human endogenous glycans, including sialoglycans and tumor-associated carbohydrate antigens. Furthermore, depleting IgG2 subclass from IVIG only moderately affected glycan recognition, in contrast to previous reports claiming that IgG2 is the main antibody subclass that recognizes polysaccharides.[121–127] A follow-up study on a much larger microarray with 610 printed glycans explored seven commercial sources of intravenous/subcutaneous pooled human IgG (Interact, Privigen, Hizentra, Gamunex, CytoGam, Rhophylac, Sandoglobulin). This analysis confirmed that the anti-glycan IgG repertoire is highly similar across various sources, and that IgG2 is not the sole subclass responsible for glycan recognition. These IgG recognized microbial glycans, as well as host glycans to which various pathogens or exotoxins bind. There was low recognition of sialoglycans and endogenous ligands for immune receptors (selectins, C-type lectins, and human siglecs). Sandoglobulin exhibited a slightly different profile compared to the other IgG pools, and when depleted of IgG2 it showed a ~66% reduction in anti-glycan reactivity, particularly to tumor-associated carbohydrate antigens and to viral or bacterial attachments sites, suggesting partial IgG2-dependence.[128] Others have used pooled Ig (IgM/A/G) to investigate IgM binding to *O*-sulfoglycans.[129]

IVIG was also examined on much smaller arrays with narrower, more targeted scopes. Four IVIG (Carimune NF, Gamunex, GammaGard, Flebogamma) were tested against 20 matched pairs of glycans with terminal *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic (Neu5Gc). Neu5Ac is a self-glycan which covers all human cells and is tolerated by the immune system, while Neu5Gc is produced only in other animals and is considered a foreign and immunogenic glycan in humans.[7] Indeed, no IVIG bound to Neu5Ac-containing

Table 2 Summary of glycan microarrays tested with IVIG/Pooled human Ig[a]

Number of glycans	Glycan types	Sample source	Serum dilution or concentration	Glass slide surface	Isotype	Ref.
377	CFG v3.1 synthetic and natural glycans	IVIG (Sandoglobulin, Privigen, Gamunex)	180 μg ml^{-1}	NHS	IgG	120
610	CFG v5.1 synthetic and natural glycans	IVIG/SCIG (Intratect, Privigen, Hizentra, Gamunex, CytoGam, Rhophylac, Sandoglobulin)	180 μg ml^{-1}	NHS	IgG	128
40	Neu5Ac/Neu5Gc-sialoglycans	IVIG (Carimune NF, Gamunex, GammaGard, Flebogamma)	1, 0.5 mg ml^{-1}	Epoxide	IgG	130
40	Neu5Ac/Neu5Gc-sialoglycans	1 serum; affinity purified anti-Neu5Gc IgG (from GammaGard IVIG)	1 : 100; 25 ng μl^{-1}	Epoxide	IgG	132
62	Neu5Ac/Neu5Gc-sialoglycans	IVIG (GammaGard, GammaPlex, Privigen, Hizentra, Immunovenin-Intact), pooled human IgA (2 lots)	1, 0.5, 0.25, 0.125 mg ml^{-1}	Epoxide	IgG, IgA	131
412	Glycoproteins, neoglycoproteins	Pooled human IgA, IgM, IgG, IgG1, IgG2, IgG3, IgG4	200 μg ml^{-1} IgG, 50 μg ml^{-1} IgA, 50 μg ml^{-1} IgM	NHS	IgG, IgA, IgM	106
421	Synthetic glycans, especially *O*-sulfoglycans	Complex Immunoglobulin Preparation (IgG/IgM/IgA)	1 mg ml^{-1}	NHS	IgM	129

[a] *N*-Hydroxysuccinimide (NHS); Consortium for Functional Glycomics (CFG).

glycans, as reported on other arrays. However, there was strong reactivity against the matched set of Neu5Gc-containing glycans, suggesting a highly specific anti-Neu5Gc IgG response in humans.[130] When various commercial IVIG (GammaGard, GammaPlex, Privigen, Hizentra, Immunovenin-Intaxt) were examined against a larger array of 62 sialoglycans, similar Neu5Gc-specific repertoires were found. Competition assays with free Neu5Gc or Neu5Gc-peptides further confirmed this high Neu5Gc-specificity.[131] Likewise, IgG from several individual human sera showed preferential binding to Neu5Gc-glycans over Neu5Ac-glycans,[91,96] and anti-Neu5Gc IgG that were affinity-purified from IVIG demonstrated extremely high Neu5Gc-specificity.[130,132–134] Neu5Ac is the precursor for Neu5Gc synthesis by CMP-Neu5Ac hydroxylase (CMAH) in most non-human mammals, and the two glycans differ by only an additional hydroxyl group in Neu5Gc.[135] The ability of serum antibodies to recognize such minute variations—as small as an individual oxygen atom—between glycans is remarkable, as is the microarray technology employed to study such trends.

In contrast to IgG, examination of pooled human IgA against the microarray of 62 sialoglycans revealed not just binding to Neu5Gc-glycans, but also cross-reactivity against several Neu5Ac-glycans, suggesting potential autoreactivity to these self-glycans.[131] Interestingly, after removing terminal sialic acids from these array-printed glycans by an on-slide sialidase treatment, there was a dramatic increase in binding of both IVIG and pooled IgA. Following this treatment, both pools recognized multiple non-sialylated glycans. Altogether, these data suggest that serum IgG and IgA could compete for the same glycan targets and emphasize the role of sialic acids as self-associated molecular patterns[136] that shield against potential attacks *via* antibody binding to self-glycans.[131]

3.3. Variability of anti-glycan antibodies over time

A key factor when evaluating serum anti-glycan antibodies is biological variability over time. Some variability is expected, even in the absence of exposure or infection. Variability can arise from changes in total antibody levels or from fluctuations in individual subpopulations. If one is identifying changes to the repertoire that are relevant to a specific disease or condition, it is critical to understand normal levels of fluctuation. For example, one might profile serum samples collected before and after acquiring a disease or before and after vaccination. To identify real responses, one must establish the normal levels of change that occur over that same period of time in the absence of disease or vaccination.

Several studies have evaluated variability of serum anti-glycan antibodies over time.[105,137] Serum anti-glycan antibody levels to a diverse assortment of glycans were found to be highly stable over several weeks.[108] For time periods of 5–10 weeks, for example, changes of 4-fold or greater were observed very infrequently ($<1\%$ of the time). Over time frames of 1–3 years, fluctuations were larger but still relatively stable, with IgG and IgM changes of 4-fold or greater occurring only 4–5% of the time. Whether considering time frames of weeks or years, antibody levels appeared to be relatively steady, rather than substantially changing in composition. No particular anti-glycan antibody subpopulation was observed to be especially variable relative to the others.

3.4. Genetic and environmental factors affecting healthy anti-glycan antibody repertoires

When comparing serum samples from experimental and control subjects, various genetic and environmental factors—such as age, sex, smoking status, country of residence, diet, and exercise—may influence numerous anti-glycan antibody populations. The ability to match cases and controls is crucial for making fair comparisons between antibody profiles and identifying antibody subpopulations that are truly specific to the cases. Therefore, information about how these factors influence antibody repertoires is helpful for designing experiments and provides insight about the development of the human immune system. Despite its value, the link between many of these factors and anti-glycan antibody profiles has been understudied.

Blood type is the best-studied factor that influences endogenous anti-glycan antibodies. Individuals produce antibodies to blood group antigens that are not present on their own cells, and antibodies to blood group A and blood group B correlate significantly with blood type. In addition, other antibody subpopulations correlate with blood type, such as antibodies against α-Gal and Forssman antigen, both of which are structurally related to blood group antigens.[138]

Age and ethnicity also influence anti-glycan antibody repertoires.[108,111,138] Luetscher *et al.* examined IgG and IgM from 105 normal sera using a designer array with 99 different glycans; a subset ($n = 8$) was also screened on two other glycan arrays, which collectively contained >1000 glycans. They concluded that anti-glycan antibodies are associated with ethnicity and age, and that each individual's repertoire of glycan-binding antibodies is relatively unique.[111] This points to a potential role for personalized profiling in precision medicine. In another study, IgM reactivity against many glycans was observed to decline with age (ages 20–65), while IgG remained relatively stable.[138] It was also shown that levels of IgG specific for certain *N*-acetyl-lactosamine (LacNAc)-containing glycans correlated with race.[138]

Similarly, diet is thought to play an important role in shaping the glycan-binding antibody repertoire. While the relationship between diet and the generation of anti-glycan antibodies is not well understood, there are some established connections between the two. One such example is Neu5Gc, as discussed in Section 3.2. Humans are exposed to this antigen through consumption of mammalian-derived foods, like red meat and dairy. Recently, dietary intake of Neu5Gc by roughly 20 000 individuals was catalogued *via* 24-hour diet questionnaires. This information, in tandem with serum profiling of 120 representative individuals (following high, medium, or low Neu5Gc-containing diets) on glycan microarrays, showed that high Neu5Gc diets resulted in higher levels of anti-Neu5Gc IgG. However, Neu5Gc consumption did not affect IgG affinity to Neu5Gc-glycans.[96]

4. Infectious disease

The glycans expressed by infectious organisms—such as viruses, bacteria, fungi, protozoan parasites, or parasitic helminths—may elicit production of anti-glycan antibodies. These responses may be deleterious to the infected human or animal host, as these antibodies may recognize not only the intended infectious antigen, but also structurally similar host glycans. This cross-reactivity is important in inducing autoimmune pathologies, as seen in Guillain–Barré syndrome[139,140] and Lyme disease.[78] In recent years, glycan microarrays have helped identify both anti-glycan antibodies associated with infectious diseases as well as potential antigens for vaccine development. They have also been extensively used to profile antibody responses induced by microbial vaccines. The studies cited below are representative of the areas of ongoing research in which glycan microarrays play a key role in analyzing immune responses to infections or vaccines.

4.1. Antibodies to bacterial glycans

Many studies have employed microbial glycan arrays in which natural or synthetic glycans representing bacterial surface carbohydrate antigens are immobilized in order to explore antibody responses to specific glycans.[141–149] The development of glycan microarrays with microbial glycans has accelerated in recent years, providing a unique resource to study human and animal responses to microbial glycan antigens and to characterize the exact glycan determinants recognized by antibacterial monoclonal antibodies (mAbs). Many of the bacterial glycan antigens targeted by the immune system are critical components of bacterial cell walls, such as peptidoglycans, capsular polysaccharides, lipopolysaccharides, and teichoic acids.[15]

4.1.1. Gram-positive bacteria. The Gram-positive bacterium *Staphylococcus aureus* colonizes 30% of people asymptomatically but can cause severe disease in some people. Thus, antibodies targeting *S. aureus*, including *S. aureus* glycans, are of great interest. Two such *S. aureus*-targeting antibodies isolated from humans, mAb 4461 and mAb 4497, were previously determined to bind to wall teichoic acids (WTAs). WTAs are peptidoglycan cell wall components characteristic of Gram-positive bacteria that are comprised of repeating ribitol-5-phosphate (RboP) or glycerol phosphate (GroP) residues. One recent study interrogated specific recognition motifs on RboP-based WTAs, which can be modified on their C-2 position with α- or β-GlcNAc and on their C-4 position with esters of D-alanine. The elegant approach of using glycan microarrays—coupled with X-ray crystallography, NMR spectroscopy, and computational studies—revealed that both mAbs recognized the RboP and the GlcNAc residues, and that mAb 4461 specifically recognized α1-4-GlcNAc-modified WTA, while mAb 4497 recognized β1-3-/β1-4-GlcNAc-modified WTAs.[150]

Complementary studies have investigated GroP-based WTAs, which are also found in the cell walls of *S. aureus* and other enterococcus and staphylococcus species. A recent study used microarrays comprised of various synthetic WTA glycans to identify the recognition motif of a mAb (WH7.01) generated by immunization of mice with WH7, a synthetic version of a GroP WTA hexamer.[151] Screening of WH7.01 with these WTA microarrays—in combination with ELISA, SPR analyses, and STD-NMR spectroscopy—found that this mAb recognized GroP-based WTA fragments but not similar RboP-containing WTAs.[151] This indicates that the GroP moiety is the main recognition element for this anti-WTA mAb.[151]

Glycan microarrays have also been used to assess antibody recognition of the capsular polysaccharides (CPSs) of the Gram-positive bacteria *S. pneumoniae*[152–155] *Clostridium difficile*, and *Bacillus anthracis*.[148,156,157] The CPSs of *S. pneumoniae*, which serve as a critical virulence factor, vary between serotypes. Existing vaccines target at most 24 of the nearly 100 *S. pneumoniae* serotypes, and a major goal of *S. pneumoniae* vaccine development is to develop more broadly protective vaccines. Glycan arrays have helped address this challenge by revealing the exact CPS motifs that are critical for antibody recognition. For example, glycan arrays were used to profile rabbit polyclonal serum after immunization with *S. pneumoniae* Serotype 4 CPS, a component of the widely used Prevnar 13 vaccine. The authors conclude that pyruvate modification of the carbohydrate antigens was key for recognition by serum antibodies and should be incorporated into synthetic Serotype 4 CPS vaccines.[153] In another study, a glycan array comprised of various CPS epitopes was used to identify a recognition motif shared between the 19A and 19F serotypes.[153]

In a study of the immune response to *C. difficile* CPS, Oberli *et al.*[156] produced a synthetic hapten of the *C. difficile* CPS PS-II. Not only did this synthetic glycan elicit a strong antibody response in mice when conjugated to diphtheria toxoid, but the authors also observed that IgA in stool from *C. difficile* infected patients bound this antigen on a glycan array.[156] Serum samples from *C. difficile* patients have also been profiled for antibodies to various oligosaccharide fragments of *C. difficile* CPS PS-I, and the best oligosaccharide was tested as a vaccine candidate.[157]

In another *C. difficile* vaccine effort, Broecker *et al.*[158] developed a synthetic, pentavalent immunizing agent containing five disaccharides, each a different minimal glycan epitope found in PS-I. After immunizing mice with this antigen, the authors observe a PS-I-specific IgG response *via* glycan microarray analysis.

Lipoteichoic acid (LTA), also known as surface polysaccharide-III (PS-III), has also been investigated as a candidate for *C. difficile* vaccination. A challenge in studying the immune response to these *C. difficile*-associated glycan epitopes is their availability: PS-I, -II, and -III are only expressed at low levels on the bacterial cell surface, forcing researchers to chemically synthesize these compounds.[159] Martin *et al.* established one of the first methods to synthesize PS-III repeating units.[159] This newly synthesized glycan was incorporated into an LTA microarray and used to profile the glycan-binding antibody repertoire in *C. difficile*-infected patients. The profiling studies were performed with the goal of identifying novel epitopes for *C. difficile* vaccine development.

Similarly, Broecker *et al.*[160] used a microarray of synthetic LTAs to screen serum from *C. difficile*-infected patients and observed LTA-specific binding by serum antibodies. This demonstrated that antibodies naturally induced by *C. difficile* infection can recognize synthetic LTAs. With this information, the authors developed a PS-III glycoconjugate using their synthetic LTAs, which was used to immunize mice. The serum antibodies isolated from these mice were screened on the synthetic LTA glycan microarray, and LTA-specific binding in serum samples from mice immunized with the PS-III glycoconjugate was observed. As a follow-up study, Broecker *et al.* tested the ability of PS-III glycoconjugate immunization to limit *in vivo C. difficile* colonization. Vaccination of mice with PS-III glycoconjugate prior to *C. difficile* challenge significantly reduced *C. difficile* colonization relative to controls.

4.1.2. Mycobacteria. Although *Mycobacterium tuberculosis* can be classified as a Gram-positive bacterium, it has a distinct set of cell wall glycans, including α-glucan and arabinomannan (AM), which can be attached to lipids to form lipoarabinomannan (LAM). Because AM is comprised of arabinofuranose residues not synthesized by human cells, AM from *M. tuberculosis* is known to be highly immunogenic in the contexts of both vaccination and infection. Glycan array profiling of serum from *M. tuberculosis*-infected individuals by Chen *et al.* revealed that the antibody response targets a diverse range of AM motifs. However, compared to IgG from symptomatic patients, the IgG from asymptomatic individuals was reactive against a particular subset of terminal AM motifs and conferred protective efficacy against *M. tuberculosis in vivo*.[161] A similar IgG reactivity profile directed against these AM motifs was observed in serum from people immunized with the bacillus Calmette–Guerin (BCG) vaccine.[162] Glycan array screening has also been used to assess the anti-AM binding profile of monoclonal antibodies against *M. tuberculosis* isolated from infected or exposed people.[163]

Given the immunogenicity of AM and the importance of an anti-AM antibody response in preventing infection, Prados-Rosales *et al.* developed a conjugate vaccine comprised of AM from *M. tuberculosis* linked to either a *M. tuberculosis* protein or a *B. anthracis* protein. Screening of serum from vaccinated mice on an array with synthetic AM fragments demonstrated that the version of the vaccine with AM conjugated to *M. tuberculosis* protein produced a broader, more effective immune response.[164]

4.1.3. Gram-negative bacteria. Array profiling has also been applied to understand the immune response to *Acinetobacter baumannii*,[165,166] an opportunistic bacterial pathogen responsible for serious nosocomial infections. For example, printed glycan microarrays with synthetic glycans representing components of CPSs of *A. baumannii* were used to identify responses in infected patients and interrogate specificity of a mouse mAb (mAbC8).[165] The results revealed that a novel tetrasaccharide comprised of *N*-acetyl-glucosamine (GlcNAc), galactose (Gal), glucose (Glc), and acetylated glucuronic acid (GlcA) is well recognized both by infected patients' antibodies and by mAbC8, making this glycan antigen a strong vaccine candidate. Related synthetic microarray approaches have also been used to characterize IgG antibody responses to glycans of *Helicobacter pylori*, a Gram-negative flagellated bacterium that colonizes ~50% of the global population and is a risk factor for the development of various gastric pathologies, including gastric cancer, duodenal ulcer disease, and gastric ulcer disease.[167] After synthesizing the core undecasaccharide of *H. pylori* LPS and related glycans, Zou *et al.* printed these components as a microarray. Subsequent screening of serum samples from infected patients revealed the key antigenic epitope to be an α-1-6-glucan trisaccharide.[168]

H. pylori expresses many other notable glycans on its surface LPS, including Lewis X, Lewis Y, and *N*-acetyllactosamine determinants.[169] Purified LPS from various *H. pylori* strains has been coupled to nitrocellulose microarrays. Preliminary screening of previously characterized plant lectins and antibodies on these arrays confirmed that many of these fucose-binding reagents also recognize LPS glycans. The binding profiles of these lectins and mAbs also revealed that LPS from some strains expressed 1-3-D-galactans and the blood group H-type 2 sequence. Many different purified human immune receptors, including DC-SIGN and galectin-3, also bound to several LPS isolates on the array. In addition, sera from individuals infected with *H. pylori* contained IgG and IgA that bound strongly to LPS of all *H. pylori* clinical isolates.[169]

Some bacteria may present unique glycans, such as keto-deoxynonulosonic acid (Kdn), an ancestral version of sialic acid that can trigger an immune response. Kdn is displayed on surface glycoconjugates of *Haemophilus influenza*, a human commensal and opportunistic Gram-negative bacterium.[170] Anti-Kdn antibodies in human sera were interrogated using a sialic acid-glycan microarray comprised of dozens of glycans derivatized with various sialic acids and Kdn. The screening revealed that commercially available IVIG contained IgG that recognized several Kdn-terminating glycans.[170] However, IgM to such Kdn antigens was not observed in infant serum. The authors therefore conclude that these anti-Kdn antibodies are not germline encoded, but rather produced by the adaptive immune response to antigen exposure within the first year of life.

Glycan microarrays have also been used to profile samples from patients with *Neisseria meningitidis*[171] and *H. influenzae*,[172] as well as *Burkholderia pseudomallei*, and *Francisella tularensis*.[144,148,149] In the *N. meningitidis* study, the authors screened sera from recovering *N. meningitidis* patients (n = 11) and asymptomatic carriers (n = 11), as well as healthy controls (n = 15). After screening on a glycan microarray, the authors observe exclusive IgG binding to specific meningococcal antigens.[171] Arrays have also been used to study responses to *Chlamydia pneumoniae* vaccines.[154,155,173]

4.1.4 Bacterial glycan-binding proteins and infection-induced autoantibodies. Interestingly, the fimbriae of bacteria possess adhesins that enable the bacteria to bind to glycans and mediate disease. For example, *Escherichia coli* can associate with plant glycans in fresh produce, leading to outbreaks. Early glycan array studies demonstrated that the *E. coli* pilus in

serotypes O157:H7 and O18:K1:H7 adhered to plant cell wall glycans of at least arabinotriose or longer, with particular affinity for α1-5-linked L-arabinose residues.[174] More recent studies using glycan microarrays found that the F9 fimbriae of *E. coli* bind a component of the plant cell wall called hemicellulose, specifically its galactosylated xyloglucans.[175]

In order to study Lyme disease, which is triggered by infection with *Borrelia burgdorferi* bacteria, Song *et al.* employed shotgun glycomics microarray strategies with a focus on GSLs, a class of glycan targets that remains elusive because they are challenging to derivatize.[78] The authors developed a fluorescent tag for conjugation to free glycans, which were subsequently immobilized on NHS-activated slides. In the first phase of this study, the array was comprised of bovine brain-derived GSLs, which were used to screen sera from healthy controls and patients diagnosed with Lyme disease. Of the GSLs tested, one fraction was bound more strongly by IgG from Lyme disease sera compared to controls. Using mass spectrometry methods, the authors concluded that this fraction contains GD1b-lactone, which was recognized despite its low abundance in the initial GSL mixture. Not only does this study identify a glycan that potentially mediates the autoimmune reaction seen in Lyme disease, but it also highlights the advantages of cutting-edge shotgun glycan array technologies. The authors further apply this strategy to develop shotgun glycan arrays of blood group-expressing GSLs from human erythrocytes and from a cultured prostate cancer cell line, illustrating applicability to other tissues and diseases.

4.2. Antibodies to fungal glycans

The pathogenic fungus *Cryptococcus neoformans* is dependent upon its polysaccharide capsule to protect itself against the host's defense system and to enhance its virulence.[176] A major component of this capsule is the polysaccharide glucuronoxylomannan (GXM), comprised of α-1-3-mannose repeating units modified by β-1-2- and β-1-4-xylose (Xyl) branches, a β-1-2-glucuronic acid (GlcA) branch every three mannoses, and heterogeneous 6-*O*-acetylation of mannoses. This acetylation, which averages 2 acetates per mannose triad (serotype A and D),[177] is important for antibody-antigen interactions. To identify which specific antigenic epitopes within GXM are recognized by mAbs, Guazzelli *et al.* constructed a microarray of diverse GXM structures. Starting from Xylβ-1-2-Man and GlcAβ-1-2-Man, the authors synthesized di-, tri-, tetra-, penta- and hexa-saccharide thioglycoside building blocks, which then allowed for generation of 10 GXM oligosaccharide structures, ranging from di- to octadeca-saccharides.[177] These glycans were printed on both epoxide and hydrogel array slides, then used to screen seventeen GXM-specific mouse mAbs, some of which were previously determined to be protective against *C. neoformans* challenge. The fact that different binding profiles were noted for some antibodies on epoxide *versus* hydrogel arrays suggests that some of the mAb recognition epitopes are conformationally dependant and may be masked depending on the slide surface chemistry. Moreover, the studies revealed that all GXM-neutralizing mAbs bound to a particular decasaccharide, supporting efforts to develop this GXM glycan into *C. neoformans* vaccines.

Recently, novel FRET-based and glycan microarray-based technologies have been combined to map the epitopes of mAbs against the capsule of *C. neoformans*.[178] For such work, the researchers prepared glycan-based FRET probes to identify antibodies with unusual innate glycosidase activity, which could then be analyzed kinetically. Several catalytic antibodies were discovered with the ability to degrade intact capsule and increase phagocytosis of cells. These exciting discoveries suggest that catalytic properties of antibodies may be important in responses to fungal infections.

4.3. Antibodies to parasitic glycans

4.3.1. Antibodies to trematode glycans. Many studies have employed glycan microarrays to examine the host immune responses to glycans of the parasitic trematode *Schistosoma* spp. Schistosomes are blood flukes and infect many mammalian hosts, including humans.[179] They are largely represented by three major species, *Schistosoma mansoni*, *Schistosoma japonicum*, and *Schistosoma haematobium*. Many mammals, including humans, are susceptible to repeated and chronic infections. While the drug praziquantel may be required to kill the parasites, recurrent infection can also lead to the development of some protective immunity against schistosomes, including an antibody response to their unusual glycans. The glycomes of schistosomes include diverse *N*- and *O*-glycans—many of which contain atypical linkages and sequences—displayed on surface/secreted glycoproteins and GSLs. Examples are glycans with the lac-di-NAc motif (GalNAcβ1-4GlcNAc; LDN) and highly fucosylated glycans, such as motifs of Lewis X, repeating Lewis X, and fucosylated lac-di-NAc (GalNAcβ1-4(Fucα1-3)GlcNAc; LDNF).[179] Schistosomes can also generate unusual GlcA/GalNAc-containing polysaccharides termed circulating anodic antigens, which are highly antigenic.[180] Interestingly, glycans of these parasites are devoid of sialic acid, as worm species do not have the genes encoding sialic acid biosynthesis and utilization.

Serum antibodies to these parasitic glycoconjugates have been studied using glycan microarrays both in defined formats (with chemo-enzymatically synthesized glycans) and in natural formats. For example, profiling sera from schistosome-infected Ghanaian children (n = 60) on a microarray of 126 synthetic *N*-glycans revealed IgE antibodies against glycans with core α2-Xylose (CX) and core α3-Fucose (CF).[181] In subsequent experiments focusing on IgG instead of IgE, sera from schistosome-infected individuals in Uganda (n = 41) were screened against arrays containing both native schistosome glycans and synthetic *N*-glycans. Antibodies of all IgG subclasses were found against a wide variety of *N*-glycans with CX and CF epitopes, as well as against terminal LDN sequences.[182] The identification of IgG4 against schistosome glycans is significant because prior work suggested that high levels of IgG4 to schistosome antigens may compete for IgE binding, limiting the IgE-mediated allergic response.[183]

Glycan microarrays have also proven valuable to understanding how the immune response to schistosome infections

varies with context, including age and host species. As a result of repeated schistosome infections over time, the immune response to schistosomes glycans changes with age. Using a defined glycan microarray to compare IgG in pooled serum from a cohort of *S. mansoni*-infected children to a cohort of infected adults, Brzezicka *et al.* demonstrated that the different age groups recognized different *N*-glycan core modifications.[184]

Also of interest are schistosome infections in animals that mount more effective immune responses than humans. While humans and many primates are vulnerable to serious schistosome infections, Rhesus monkeys and the brown rat (*Rattus norvegicus*) are much more resistant to chronic infections. While serum screening on defined microarrays identified IgG against CX, CF, and LDN-based epitopes in both rhesus monkeys and humans infected with schistosomes, the titers of antibodies against these glycans were much higher in infected rhesus monkeys than in infected humans.[185] Rhesus sera with elevated antibodies to CX, CF, and LDN were shown to be lethal to schistosomula *in vitro*.[185]

More recent microarray studies have investigated *S. mansoni* infection in the brown rat (*Rattus norvegicus*). In accordance with the aforementioned studies on human and rhesus sera, *S. mansoni* infection in the brown rat induces strong IgG titers to a non-mammalian-type modification by CX and CF epitopes.[186] The surfaces of both young schistosomula and adult worms contain high levels of glycoproteins carrying these epitopes, as evidenced by deconvolution microscopy. Most importantly, IgG that bind these epitopes can kill schistosomula by a complement-dependent process *in vitro*. Such results suggest the need to explore the potential of novel glyco-based vaccines containing CX/CF epitopes.

A recent study investigated this potential for vaccines against schistosomes by examining the IgG and IgM responses in baboons vaccinated with irradiated cercariae (larval trematode)[187] over the course of 25 weeks. Using glycan microarray analyses, the results demonstrated that anti-glycan IgM responses to *O*-glycans, polyfucosylated antigens, and GSLs developed early after vaccination without rise afterward with subsequent revaccinations, whereas the anti-glycan IgG developed more slowly, but was elevated by subsequent vaccinations. Beyond the studies discussed here, various others have similarly employed glycan microarrays in service of identifying vaccine candidates and developing vaccines against trematode parasites, as well as screening serum from trematode-infected patients.[55,188–191]

4.3.2. Antibodies to nematode glycans. Nematodes pose a significant public health burden in the developing world; children are often chronically infected, causing malnutrition and stunting cognitive and physical development.[192] Though related to the trematode parasitic helminths such as *Schistosoma* spp. described above, nematode *N*- and *O*-glycans and GSLs are somewhat distinct. For example, an unusual glycan modification characteristic of many nematodes is 6-substitution on GlcNAc residues with phosphorylcholine (PC) or phosphoethanolamine. In order to screen for antibodies against these unique glycosylation motifs, mono- and disaccharides were synthesized with these modifications, coupled to BSA, and printed in microarrays. Profiling experiments revealed that these glycan antigens were recognized by IgG and IgM in sera of dogs and pigs,[193] including those infected with *Dirofilaria immitis*, *Ascaris suum*, and *Oesophagostomum dentatum*.[193] Dogs infected with *A. suum* have also been found to possess IgM and IgG antibodies to a wide variety of other nematode glycans, including those with motifs involving LDN, LDNF, glucuronylation, polyfucosylation and unusual chitobiose-containing glycans.[194] Some of these antigenic epitopes are shared by Schistosoma species.

Haemonchus contortus is a parasitic nematode with glycans containing CF motifs, which are recognized by IgE of infected sheep.[195] These antibodies seem to mediate a protective response, as lambs vaccinated with excretory/secretory (ES) glycoproteins from *H. contortus* were found to be immune when subsequently challenged with *H. contortus*. Analysis of the pooled lamb serum using a 264-component glycan microarray from the Consortium for Functional Glycomics (CFG) revealed that vaccination induced IgG against Galα1-3GalNAc and GalNAcβ1-4(Fucα1-3)GlcNAc.[196] While this novel Galα1-3GalNAc motif had not previously been identified in helminths, further experiments demonstrated that common α1-3-galactosyltransferases can modify the LacdiNAc disaccharide to generate the Galα1-3GalNAc epitope *in vitro*.[197]

Recently, a natural glycan microarray was constructed from 136 different glycan species isolated from the model organism *Caenorhabditis elegans.*[198] Characterization using chromatography, mass spectrometry, and lectin binding assays identified core glycan structures found in many nematodes, as well as many unusual high mannose, pauci mannose, high fucose, and PC-substituted glycans. Because of the similarities in glycan structures and motifs among nematodes, and because anti-glycan toxins that bind *C. elegans* also bind *H. contortus*, *A. suum*, and *O. dentatum*, this array constructed from *C. elegans* may prove useful for other species as well. It may also be applied to broadly identify carbohydrate-binding proteins in other nematodes, as well as insects and trematodes.

Petralia *et al.* printed natural glycan microarrays containing *N*-glycans and GSL-derived glycans from the parasitic nematode *Brugia malayi* on epoxy-coated glass slides.[199] Some of the unusual *N*-glycans from *B. malayi* contain PC-substituted GlcNAc and PC-substituted mannose residues, along with unusual terminal α-Gal, GalNAc, GlcNAc, and GlcA residues. Interestingly, these glycans lack the CF motif commonly seen in other nematodes. Probing the array with sera from *B. malayi*-infected macaques identified IgG that recognized a large number of *N*-glycans, along with most of the GSLs. Similarly, screening of sera from *B. malayi*-infected humans demonstrated the presence of IgG to many of the parasite-derived *N*-glycans and GSLs. This *B. malayi* glycan microarray was also used to screen plasma from individuals infected with the related filarial nematodes *Loa loa*, *Onchocerca volvulus*, *Mansonella perstans* and *Wuchereria bancrofti*.[200] It was observed that IgG from all infected plasma bound to a subset of cross-reactive *B. malayi* glycans. Array studies allowed the identification of

both broadly cross-reactive and specific glycan targets for each nematode infection, providing new insights into potential diagnostics and disease control.

Glycan microarrays have also been used to probe the repertoire of glycan-binding antibodies from patients with a variety of other nematode infections, such as Trichinella.[201]

4.3.3. Antibodies to malaria. It has been established that people living in malaria-endemic regions frequently develop antibodies targeting parasite glycosylphosphatidylinositol (GPI), a toxin with important roles in malaria pathogenesis. To better understand this anti-GPI antibody response, Kamena *et al.* printed a synthetic glycan microarray of seven GPI glycan fragments with zero to four mannose residues linked to a glucosamine-inositol core. Screening serum samples from subjects exposed to malaria revealed little binding to glycans shorter than a pentasaccharide, but significant IgG against the four longer GPI fragments with at least three mannose residues.[202] Thus, the authors concluded that a pentasaccharide is the smallest epitope that can induce a natural anti-GPI antibody response.

The authors also interrogate the effect on antibody recognition of a GPI glycan with three *versus* four mannose residues. Serum antibodies from malaria-endemic regions recognized both Man3-GPI and Man4-GPI glycan fragments, but IgG targeting Man4-GPI epitopes were observed at higher levels. This indicates that the fourth mannose likely plays a critical role in antibody recognition of GPI. Such information regarding the immunogenicity of Man4-GPI alone and in relation to Man3-GPI may prove important in future development of a carbohydrate-based antitoxin vaccine for Malaria, and the serum antibodies against these glycans may act as potential biomarkers for malaria exposure.

Beyond profiling serum from people living in malaria-endemic regions, the GPI-based glycan microarrays were also used to identify the presence of anti-GPI antibodies in unexposed individuals. Profiling such sera has allowed for a heightened understanding of the natural response to malaria and parasites, potentially informing the design of future GPI-based vaccine candidates.[202]

4.3.4. Antibodies to leishmania parasites. Leishmaniasis, caused by protozoan Leishmania parasites, requires early diagnosis and intervention for effective treatment. However, current diagnostic tools yield inconsistent results depending on the host and antibody titer. Hoping to identify a more reliable diagnostic marker, Anish *et al.*[203] investigated leishmanial cell surface-displayed lipophosphoglycans (LPGs) as a potential candidate. The authors synthesized a glycan array containing four synthetic glycans, each part of the LPG found in *Leishmania donovani* and *Leishmania chagasi*. The array was used to screen sera from infected dogs (n = 50), asymptomatic dogs from Leishmania-endemic regions (n = 25), asymptomatic dogs from Leishmania-nonendemic regions (n = 20), and dogs kept in a pathogen-free setting (n = 32). For each of the LPGs tested, infected dogs demonstrated higher levels of serum anti-LPG antibodies compared to dogs from Leishmania-nonendemic regions. When comparing infected and asymptomatic dogs—both from endemic regions—there was only a significant difference in IgG against one LPG antigen (p = 0.0361). The authors also screened sera taken from patients with visceral (VL, n = 23) or cutaneous (VC, n = 6) leishmaniasis, in addition to healthy controls (n = 5). Though there was considerable variability within each group, higher levels of antibodies against two of the four LPG glycans were observed in the leishmaniasis groups compared to the controls. 95% of VL patients and 33% of VC patients tested positive for antibodies targeting a trimannan LPG trisaccharide, and 52% of VL patients and 66% of VC patients tested positive for antibodies targeting a tetrasaccharide comprised of trimannan with an additional Galβ1-4Man linkage.

Because they observed such variability between samples, the authors sought to generate a single glycan antigen capable of inducing an antibody response targeted against various leishmanial glycans. They selected the tetrasaccharide LPG antigen that included both the trimannan and Gal1-4Man epitopes identified as important recognition elements in the initial glycan array screening experiments. Following immunization of mice with this tetrasaccharide conjugated to a CRM carrier protein, strong binding to LPG components was observed by microarray analysis. The authors conclude that that antibodies against the LPG tetrasaccharide could serve as a valuable tool in *Leishmania* diagnosis.[203]

4.3.5. Antibodies to other parasites. Glycan arrays have also been used to profile antibodies to a variety of other parasites.[204–206]

In evaluating antibodies in the sera from *Toxoplasma gondii* infected patients, all patients with acute (n = 10) or latent (n = 10) toxoplasmosis possessed IgG towards a *T. gondii* GPI glycan and its fragments, whereas these antibodies were lacking in sera of the healthy controls (n = 10).[204] Sera from patients infected with *Cryptosporidium parvum* have also been studied, and microarray studies demonstrated that patient sera contained considerable IgM against the Tn antigen (GalNAcα1-Ser/Thr), which is found in the mucins of the parasite.[205]

4.4. Antibodies to viral glycans

Because so many viruses (HIV, SARS-CoV-2, *etc.*) display glycoproteins on their surfaces, serum antibodies recognizing viral glycans are interesting candidates for microarray analysis. Antibodies against SARS-CoV-2-associated glycans have been of particular interest over the last several years, and various groups have applied glycan microarray screening to understand SARS-Cov-2 immune responses and therapeutics.[207–209] Butler *et al.* compared two cohorts of SARS-CoV-2 patients to uninfected controls by profiling serum IgG and IgM on extensive glycan microarrays.[208] While antibodies from SARS-CoV-2 patients displayed elevated binding to multiple self-glycans—including gangliosides that can cause neurological disorders—overall IgM reactivity was lower in patients compared to controls. This is indicative of a global immune dysregulation, particularly of the IgM isotype. Severe disease was found to be associated with antibody binding to *N*-glycans, Forssman antigen, and ovalbumin.[208]

In another study, a glycan microarray was used to characterize several therapeutic neutralizing human mAbs that recognize glycosylated epitopes on the spike receptor-binding domain of SARS-CoV-2.[209] Serum antibody reactivity to SARS-CoV-2 has also been studied longitudinally, demonstrating the utility of microarray technology in providing a more comprehensive understanding of SARS-CoV-2 immunogenicity.[207] Interestingly, long before the SARS-CoV-2 pandemic, Wang *et al.*[210] also leveraged glycan microarrays to investigate the glycan-binding antibodies generated in response to an inactivated vaccine comprised of the first SARS-CoV virus. The authors observed antibody binding to a self-glycoprotein, asialo-orosomucoid, suggesting that the SARS-CoV virus may share similarity in glycosylation to that seen on some healthy human cells.[210]

Anti-glycan antibody responses to HIV have also been investigated using glycan arrays. While nearly 38.5 million people worldwide were living with HIV as of 2021, there is no vaccine or cure for HIV-1. Development of such clinical assets would be aided by a deeper understanding of the broadly neutralizing antibody (bnAb) response observed in some HIV-infected individuals, which is often directed against viral glycans. Scheepers *et al.* interrogated this response by using a 245-component glycan microarray composed of *N*-linked carbohydrates, glycolipids, and Thomsen-nouveau (Tn)-peptides to longitudinally screen sera from women with (n = 20) and without (n = 20) HIV. Twelve women had bnAbs against HIV glycoproteins.[211] Serum profiling revealed that sera from HIV-positive women displayed elevated binding to 40 array components. Furthermore, bnAb-generating women had especially high levels of particular glycan-binding IgGs. The authors theorized that this may constitute a phenotype useful in designing an HIV vaccine candidate. Glycan arrays have also been used to evaluate responses to SIV vaccines[212] and HIV vaccines.[213–216]

5. Cancer

Major changes in glycosylation occur during the onset and progression of cancer, including decreased expression of normal glycans and increased expression of tumor-associated carbohydrate antigens (TACAs), which have little or no expression in healthy adult tissue. Prominent examples of TACAs include sialyl Lewis A (sLeA, the CA19-9 antigen), Lewis Y (LeY), GD2, GM2, GD3, fucosyl GM1, Globo H, Tn, sialyl Tn (sTn), the Thomsen–Friedenreich antigen (TF), and polysialic acid (Fig. 1).[217] Some of the most-studied TACAs are found on the mucin family glycoproteins, which are known to be both overexpressed and aberrantly glycosylated in cancer.[218,219]

Changes to the glycome can trigger immune responses against tumor antigens, immune suppression by the tumor, or sequestration of existing serum antibodies *via* tumor binding. Thus, the modified cancer glycome can affect serum antibody profiles, altering the levels of existing antibodies or inducing the development of new antibodies. Analyzing serum antibodies using glycan arrays therefore provides valuable information about the immune response to tumors, the presence or absence of various tumor antigens, and the effects of different treatments.

5.1. Early detection and diagnosis

For many types of cancer, early diagnosis is one of the most important determinants of patient outcome. There is an urgent need for screening strategies that can detect disease earlier and less invasively, when cancer is often missed or misdiagnosed. Many existing serum biomarkers lack sensitivity and specificity, and either fail to detect early-stage disease or fail to distinguish between malignancies and other diseases. Improved clinical performance may be achieved both by identification of newer, better biomarkers and by combining existing biomarkers into panels. Glycan microarrays allow for the evaluation of many different antibody subpopulations simultaneously: this technology holds tremendous potential for identifying both individual anti-glycan antibody biomarkers and panels of biomarkers. Studies in this area are summarized in Table 3.

5.1.1. Breast cancer. Soon after the development of glycan microarrays in the early 2000s, they were applied to serum screening for cancer diagnosis. Huflejt *et al.* analyzed serum from patients with metastatic breast cancer using a diverse array of over 200 glycans. They identified widespread increases in antibodies to a range of glycans and glycoproteins, including known TACAs.[220] Since then, many groups have used more targeted glycan arrays to investigate the presence of antibodies to specific glycan families in cancer patients.

One such example are *O*-glycopeptide arrays focused on glycosylated mucin peptides. The mucin family glycoprotein MUC1 (also known as CA15-3) is over-expressed and aberrantly glycosylated in cancer. While the serum level of MUC1 itself has been used as a biomarker for breast cancer,[221] it has very low sensitivity as a diagnostic for early-stage disease. An appealing alternative is the antibody response to mucins. Because mucins are typically recognized as self-epitopes, antibodies to these targets are rarely generated. However, the strong antibody responses generated against injected Tn-MUC1 in both mouse models and in human cancer patients[222,223] are evidence that immunological tolerance is overridden with regard to the altered mucin glycosylation observed in cancer.[218] These anti-mucin antibodies are highly specific for a particular mucin glycopeptide, as opposed to a glycan or peptide alone.[224]

In one early screening study for anti-mucin antibodies, Wandall *et al.* used their array of glycosylated and non-glycosylated forms of 60mer MUC1 and MUC2 glycopeptides to screen sera from recently diagnosed breast, ovarian, and prostate cancer patients (n = 56) alongside healthy controls (n = 39).[218] While IgG in healthy controls did not bind any mucin glycopeptides, serum from several cancer patients contained IgG reactive to Tn-, sTn-, and core 3-modified MUC1. This confirmed that antibodies to MUC1 peptides with truncated glycosylation can arise in anti-cancer immune responses.

This preliminary profiling was extended to characterize serum antibody binding in a larger cohort of breast cancer patients against an array consisting of a single 20mer MUC1

Table 3 Summary of glycan microarray studies for early detection and diagnosis of cancer

Cancer type	Array composition	Samples screened	Key findings and glycan antigen hits	Ref.
Breast cancer	~200 glycans and glycoproteins	18 patients, 15 healthy controls	LacNAcs, Fuc-Core-2s, Sulfo-Core-2s, sTn Antibodies to these glycans were significantly higher in patients compared to healthy controls	220
Breast, ovarian, prostate	15 glycosylated/non-glycosylated MUC1 and MUC2 peptides	56 patients (ovarian, breast, prostate), 39 healthy controls	Tn-, sTn-, and Core 3-modified MUC1 IgG to these antigens was present in several cancer patient serum samples but absent in all healthy controls.	218
Breast cancer	31 glycosylated/non-glycosylated MUC1 peptides	395 patients, 108 benign breast disease controls, 99 healthy controls	Core 3-MUC1, sTn-MUC1 More then 30% of cancer patients demonstrated antibody binding to these antigens; whereas less than 15% of healthy controls yielded the same result. IgG levels correlate with tumor grade.	224
Breast cancer	11 glycosylated/non-glycosylated MUC1 and MUC2 peptides and proteins	Total: 1036 patients, 1368 controls Discovery: 273 patients, 273 controls Validation: 431 patients, 431 controls Third set: 332 patients, 664 controls	MUC1-Core 3, MUC1sTn, MUC1Tn Antibodies to these antigens were observed to be elevated in cancer cases in the discovery set (however, the same trend was not observed in the validation set)	219
Breast cancer	40 sialylated glycans (Neu5Ac *vs.* Neu5Gc)	Discovery set: 87 patients, 25 healthy controls Validation set: 74 patients, 25 controls	sTn with Neu5Gc sialylation Antibody signals to this glycan demonstrated predictive power (AUC = 0.6 in ROC analysis) for breast cancer detection.	132
Breast cancer	8 Globo H and analogs	58 patients, 47 controls	Globo H IgG and IgM levels in cancer patient sera were roughly twice as high compared to healthy controls.	228
Ovarian cancer	203 synthetic glycans (mammalian, bacterial, and cancer-associated motifs)	33 patients, 24 healthy controls	P_1 and sialyl 6-sulfo LacNAc, 6-sialyl LacNAc, LN-6′LN, sTn, 6-sulfo-3′-sialyl TG Low antibody levels to this combination of antigens served as a better diagnostic (79.2% sensitivity and 84.8% specificity) than the standard in the field.	230
Ovarian cancer	203 synthetic glycans (mammalian, bacterial, and cancer-associated motifs)	74 patients, 81 controls	P blood group glycans (P_k, P, and P_1) Anti-P_1 IgM is lower in cancer cases and is correlated with stage. P glycans were identified on glycosphingolipids in cancer tissue samples.	231
Ovarian cancer	3 glycans (Globo H + 1 positive control + 1 negative control)	19 patients, 29 controls	Globo H IgG/M to this antigen, when used in tandem with CA-125 levels, improved diagnostic accuracy when compared to the standard (CA-125 alone).	232
Ovarian cancer	22 sialylated and sulfated glycans	22 patients, 31 controls	sTn, 6-*O*sulfo-TF, 6-*O*sulfo-LacNAc, sLeA, GM2 Classification based on low antibody levels against these glycans, alongside CA-125 levels, detected almost all cancer cases (AUC = 0.9853 in ROC analysis).	233
Colorectal cancer	Array 1 (“glycochip”): 7 glycan antigens + 3 anti-human antibodies Array 2 (“oncochip”): 6 anti-glycan antibodies	33 patients, 67 healthy donors, 27 IBD patients	Tn, TF, sLeA, Manβ1-4GlcNAc Antibody levels against these glycans combined with overall immunoglobin level and several known tumor markers correctly classifies 93% of cases.	235
Colorectal cancer	51 glycan antigens (various types)	44 patients, 53 healthy controls	3′-*O*-sulfo-LeA and 3′sialyl-TF Antibodies to these antigens identified 74.3% of cancer cases in the cohort.	236
Colorectal cancer	49 mucin glycopeptides and glycoproteins	58 patients, 39 IBD patients, 50 + 53 healthy controls	Tn-, sTn-, and Core 3-modified MUC1 IgG to these antigens was observed in ~75% of screened cancer serum samples.	237
Colorectal cancer	62 sialylated glycans (Neu5Gc *vs.* Neu5Ac)	71 patients, 71 healthy controls	Neu5Gc-containing glycans High cumulative IgG signal to Neu5Gc-containing glycans was associated with incidence of colorectal cancer.	137
Prostate cancer	64 *N*-glycans (glycoproteins, synthetic glycans, glycolipids, non-human glycans)	17 patients, 12 controls	Man-9 Levels of antibodies to these glycans were significantly higher in cancer patients relative to controls	241

Table 3 (*continued*)

Cancer type	Array composition	Samples screened	Key findings and glycan antigen hits	Ref.
Liver cancer	2 AFP glycoforms (AFP-L1 and AFP-L3)	32 patients, 9 hepatitis B patients	AFP-L3 (fucosylated) Antibody signals to this antigen were significantly higher in patients compared to controls.	245
Liver cancer	58 synthetic glycans	293 patients, 133 hepatitis B patients, 134 hepatitis C patients, 33 healthy controls	Sialyl LacNAc $6SO_3$, DSGG, Fuc-GM1, Gb3, Gb2, B19, and Man7 IgG levels to these antigens were significantly elevated relative to controls	246
Lymphoma	37 human and plant glycans	8 patients, 8 healthy controls	L-Araf, Fuc(α), Fuc(α1-2)Gal-β, Tn, and GalNAc(β) Antibody signal to these glycans was elevated compared to in controls	249
Gastric cancer	300 glycan antigens (various types)	146 patients, 55 healthy controls	KDNβ1-6′LN-C3, β1-3′SLN, LN-C8, Aα1-4A, TF, 3′Sialyl-LeC and Tn3Su IgM to these antigens in cancer signature. GN6Su, TF, para-Fs and βGU IgG to these antigens in cancer signature.	252
Pancreatic cancer	609 glycan antigens (various types)	14 patients, 6 controls	Terminal GlcNAcα1-4Galβ1-4GlcNAc This motif was present in half of the glycans with elevated IgA in pancreatic cancer.	258
OSCC	22 glycan antigens (various types)	65 patients, 21 healthy controls.	SSEA-3, GD2 IgM signal to these antigens was elevated in cancer patients relative to controls. GD2, GloboH, LeY, sLeX IgG signal to these antigens was reduced in cancer patients relative to controls.	261

tandem repeat modified with various glycosylation motifs.[224] When binding of serum from early-stage breast cancer patients (n = 395) was compared to age-matched controls with either benign breast disease or no disease, it was observed that more than 30% of the cancer patients had elevated antibody signals to core 3- and/or sTn-MUC1 peptides, compared to less than 15% of controls.

Given the impressive results in distinguishing early-stage cancer patients from healthy controls, Burford *et al.* carried out one of the largest glycan microarray studies ever conducted to investigate if anti-mucin antibodies could be detected in serum prior to cancer diagnosis.[219] The study screened over 2400 prospectively collected samples, including a discovery set from the UK collaborative trial of ovarian cancer screening bank (UKCTOCS) consisting of 273 women who later developed breast cancer matched to 273 controls. Further investigation used a validation set consisting of serum from 431 breast cancer cases and 431 matched controls from the UKCTOCS bank, as well as 332 breast cancer cases and 664 controls from the Guernsey Island serum bank. These serum samples were profiled on a small glycan array containing seven MUC1 and MUC2 60mer glycopeptides and four MUC1 glycoproteins with either no glycosylation or Tn, TF, or sTn glycans. While the discovery set identified elevated antibody signals to MUC1-core 3, MUC1STn, and MUC1Tn in cancer cases, these findings were not confirmed in the validation set. The authors also found no difference in anti-MUC1 antibodies when comparing other adenocarcinoma patients (ovarian, lung, and pancreatic cancer) to controls, ultimately concluding that serum antibodies to this set of glycopeptides are not more reliable biomarkers for earlier diagnosis compared to standard detection methods. However, given the initially promising results with early-stage disease, anti-MUC1 antibodies could still prove valuable as cheaper, faster detection methods, or as a component of a larger panel of cancer markers in monitoring disease progression.

In an alternative screening approach, Padler-Karavani *et al.* profiled serum from breast cancer patients using an array of glycans containing different sialic acid variants.[132] Although humans lack the enzyme required to synthesize Neu5Gc and generally use Neu5Ac instead, Neu5Gc from ingested animal products can be incorporated into glycans on human cells---including cancer cells. Hypothesizing that these Neu5Gc-containing glycans on cancer cells comprise novel "xeno-autoantigens" and that antibodies against these glycans could serve as novel biomarkers, Padler-Karavani *et al.* constructed an array of 20 pairs of glycans with either Neu5Ac or Neu5Gc sialylation.[132] Sera from breast cancer patients (n = 87) and healthy controls (n = 25) were screened. Using a model that quantified IgG signal to Neu5Gc glycans as a function of the printed glycan concentration, the authors found elevated signals to four glycans with predictive power for the cancer cases, each giving an area under the curve (AUC) above 0.55 on receiver-operator characteristic (ROC) curves. Of these candidate glycans, sTn with Neu5Gc sialylation performed best in a validation set of additional cases (n = 74) and controls (n = 25),

yielding an AUC of 0.6. High IgG signals to this glycan also demonstrated predictive power (AUC = 0.59) in identifying prostate, ovarian, lung, and endometrial cancers. The authors hypothesize that other novel xeno-autoantigen tumor biomarkers, such as Neu5Gc-containing Lewis X (LeX), may exist.

Other glycan arrays for breast cancer have focused on Globo H. The Globo H antigen is commonly found on GSLs and is upregulated on the surface of cancer cells, especially in breast cancer. In the 1980s, Globo H was identified as the target antigen of mAbs generated by immunizing mice with the breast cancer cell line MCF-7,[225,226] suggesting that a similar anti-Globo H antibody response might occur naturally in breast cancer patients. In 2006, Wong and coworkers confirmed the existence of these hypothesized Globo H-binding antibodies, demonstrating that IgG and IgM in serum from a breast cancer patient bound to Globo H printed on array slides.[227] Building on this finding, the Wong group screened sera from breast cancer patients on a small array consisting of Globo H and its analogs.[228] After using the array to assess the binding of several mAbs previously reported to bind Globo H and the closely-related glycan Gb5, they screened serum samples from breast cancer patients (n = 58) alongside healthy donors (n = 47). Normalizing anti-glycan antibody signals to anti-Gb5 signals, which were high in both patients and controls, they found that levels of both anti-Globo H IgG and IgM in breast cancer serum were roughly double that of healthy controls ($p < 0.0001$).[228]

5.1.2. Ovarian cancer. The existing standard for serum-based ovarian cancer screening, the mucin CA-125, has poor sensitivity: as many as 80% of women with elevated CA-125 do not actually have cancer.[229] Over the last decade, glycan microarray profiling has identified serum anti-glycan antibodies that show promise as alternative diagnostic biomarkers. In a screening study of patients with non-mucinous ovarian cancer (n = 33) compared to healthy controls (n = 24), Jacob *et al.* used a 203-component glycan array to profile serum antibody binding to a diverse set of synthetic glycans, including important mammalian, bacterial, and cancer-associated motifs.[230] Antibody levels to 24 glycans were significantly lower in the cancer patients relative to the healthy controls ($p < 0.05$). The authors attribute this trend to tumors binding and capturing these serum anti-glycan antibodies. The best-performing antigen of this set was P_1, a member of the P blood group system known to be displayed on mesothelioma cell GSLs. IgG/IgM/IgA levels against P_1 discriminated between cancer cases and controls with similar sensitivity (70.8%) and specificity (78.8%) to the standard CA-125 diagnostic.[230] Using anti- P_1 antibody levels in tandem with antibodies targeting an additional five glycans (sialyl 6-sulfo LacNAc, 6-sialyl LacNAc, LN-6'-LN, sTn, and 6-sulfo-3′-sialyl TF) further improved diagnostic sensitivity (79.2%) and specificity (84.8%).

In a subsequent study, the same 203-component array was used to screen serum from a larger cohort of ovarian cancer patients (n = 74) and controls (n = 81).[231] Anti-P_1 antibodies were primarily IgM in subclass and were again found to be present at significantly lower levels in the tumor group compared to subjects with benign or no disease. Supporting the biological relevance of antibodies to P blood group glycans, the authors identified *in vivo* P_k, P, and P_1 antigen expression on GSLs extracted from cancer tissue samples. Furthermore, they confirmed that anti-P_1 antibodies derived from patients' ascites fluid bound not only synthetic P_1 trisaccharide, but also naturally expressed cell–surface P_1 on an ovarian cancer cell line.

In addition to printed glycan arrays, a suspension glycan array that included Globo-H-conjugated fluorescent microspheres was used to screen ovarian cancer serum. The authors identified a significant decrease in anti-Globo H IgG (p = 0.009) and IgM (p = 0.071) in the high-grade serous ovarian cancer cases (n = 19) relative to controls (n = 29).[232] Incorporating both these decreased anti-Globo H antibody levels and CA-125 levels into a diagnostic classifier improved accuracy over a test based only on CA-125 levels, yielding an AUC of 0.8539 for the ROC curve. The specificity of the serum antibodies for Globo H *in vitro* was confirmed by experiments with Globo H-positive cancer cell lines.[232]

A similar suspension glycan array was used to profile plasma against 22 sialylated and sulfated glycans, many of which are linked to cancer cell metastasis.[233] Comparing plasma from ovarian cancer patients (n = 22) and donors with benign disease (n = 31), Pochechueva *et al.* found that seven glycans discriminated cancer cases from controls ($p < 0.001$), with antibody signals significantly lower in cancer serum relative to the controls.[233] Combining CA-125 levels with a set of glycan-binding antibodies (IgM to sTn, IgM to 6-*O*Sulfo-TF, IgG to 6-OSulfo-LacNAc, IgG to sLeA, and IgG to GM2), resulted in nearly complete detection of all cancer cases in this cohort (AUC of 0.9853 for the ROC curve). Moreover, the antibody signals against sTn and 6-OSulfo-TF proved particularly valuable in detecting the subset of ovarian cancer cases in which CA-125 signal was moderately elevated but not sufficiently high for accurate diagnosis.

The second-best discriminator identified was IgM against 5-*O*Sulfo-TF, a significant finding because the role of glycan sulfation in the context of cancer has not been well studied. As with breast cancer,[132] differences in reactivity to the different sialic acid variants were seen in ovarian cancer, including Neu5G-sTn *versus* Neu5Ac-sTn. While in this case anti-Neu5Gc-sTn IgG levels were significantly lower in the cancer cases relative to the controls,[233] Padler-Karavani *et al.* had previously observed that anti-Neu5Gc-sTn antibody levels were *higher* in cancer patients.[132] A possible explanation for this discrepancy is that the analysis by Padler-Karavani excludes metastatic cases, whereas the cohort examined by Pochechueva is nearly 20% Stage 4 ovarian cancer cases. It is possible that either immune suppression or the capture of antibody by tumor may decrease the levels of anti-glycan antibodies in serum from late-stage cancer patients.

5.1.3. Colorectal cancer. Typical biomarkers for blood-based screening of colorectal cancer include CA19-9 and carcinoembryonic antigen (CEA). Even when used as a pair, however, these markers are insufficiently accurate: both CA19-9 and CEA are expressed below the diagnostic threshold in nearly

60% of patients diagnosed with low-grade tumors.[234] There is an unmet need for improved biomarkers.

Butvilovskaya *et al.* used two arrays to evaluate the presence of TACAs and anti-TACA antibodies as biomarkers for colorectal cancer.[235] The first array consisted of immobilized antibodies to CEA, CA19-9, and other biomarkers. The second array consisted of seven immobilized glycans, as well as antibodies against IgG, IgM, and IgA.[235] Comparing serum from patients with colorectal cancer (n = 33) to healthy donors (n = 67) or patients with Crohn's disease or ulcerative colitis (n = 27), the authors determined that antibody levels to Tn, TF, sLeA, and Manβ1-4GlcNAc had diagnostic value. More specifically, elevated antibodies against Manβ1-4GlcNAc were the single most effective glycan predictor of a cancer diagnosis (AUC of 0.725 on a ROC curve). In addition to antibodies targeting these glycans, overall elevated levels of IgG, IgM, and IgA were found to be good diagnostic predictors for colorectal cancer (AUC of 0.663). A multi-parameter diagnostic based on overall antibody levels, anti-glycan antibody levels, and tumor protein biomarker levels correctly classified 95% of the cohort. Though many of these markers had not previously been used in cancer diagnostics, this combination of serum markers proved very effective.

In a more recent analysis of colorectal cancer, Tikhonov *et al.* screened a 51-component glycan array for IgG and IgM antibodies in serum from colorectal cancer patients (n = 44) and age- and sex-matched donors (n = 53).[236] Of the IgG signals, only antibodies against 3′-*O*-sulfo-LeA significantly discriminated between patients and controls (AUC of 0.625 on ROC curve). Additionally, IgM signals to 16 different glycan targets were elevated in the colorectal cancer patients ($p < 0.05$). The combination of IgM levels to 3′-*O*-sulfo-LeA and 3′sialyl-TF best distinguished patients from controls, correctly identifying 74.3% of cases.

Interestingly, the results of screening for anti-MUC1 antibodies in breast cancer sera largely held true in colorectal cancer serum screening as well. An array consisting of both enzymatically glycosylated mucin peptides and recombinantly expressed mucin glycoproteins was used to screen sera from colorectal cancer patients (n = 58) and controls with either inflammatory bowel disease (IBD) or no disease. IgG antibodies to three MUC1 glycopeptides (Tn-, sTn-, and core 3-MUC1) were identified in nearly 75% of colorectal cancer patients with stage I–III disease.[237] Although core 3-MUC1 autoantibodies were also present in IBD patient sera, the Tn- and sTn-MUC1 antibodies were highly cancer specific, with sTn-MUC1 IgG found in 57% of cancer patients, compared to just 10% of IBD patients and less than 5% of healthy controls.

Although serum IgM in cancer shows broad mucin reactivity,[218,237] serum IgA binding was postulated to provide insight relevant to colorectal cancer because of the significant IgA production by the large intestine. Limited IgA to MUC1 was observed in colorectal cancer patients, but there was significant IgA to MUC4, especially Tn-MUC4.[237,238]

Elevated serum IgG to Neu5Gc-containing glycans is another potential colorectal cancer biomarker and has been proposed to trigger inflammation, mediating the well-documented link between red meat intake and colorectal cancer.[239] Samraj *et al.* screened serum from colorectal cases (n = 71) and matched controls on an array consisting of 31 pairs of glycans with either Neu5Gc or Neu5Ac sialylation.[137] No association was found between elevated IgG to individual Neu5Gc glycans and cancer risk. However, when the authors looked at the cumulative signal to all Neu5Gc-containing glycans, they found that individuals in the top quartile for total IgG signal to all Neu5Gc glycans had an almost three-fold increase in colorectal cancer incidence.

5.1.4. Prostate cancer. Alterations to glycosyltransferases that occur in cancer can expose cellular *N*-glycan motifs otherwise masked by sialylation. These cryptic *N*-glycan antigens—including Man9, tri- or multi-antennary type II (Galβ1-4GlcNAc) or Tri/m-II, and Tri/m-Gn (GlcNAc)—are similar to viral glycans that trigger immune responses during HIV and SARS-CoV infection. Whole cell cancer vaccines have likewise been demonstrated to trigger production of anti-Man9 antibodies,[240] suggesting that these glycans might also induce an immune response when present on tumors instead of viruses. Wang *et al.* screened for antibodies to these *N*-glycans in serum from men with prostate cancer (n = 17) or a benign prostate condition (n = 12) using a 64-component glycan array comprised of a range of glycoproteins, synthetic glycans, glycolipids, and non-human glycans.[241] High levels of anti-Man9 antibodies were observed in both populations, but were significantly elevated in cancer serum ($p < 0.001$). A subset of cancer patients was also found to have significantly elevated IgG to OR-ASOR, a glycoprotein displaying the β1-6 branching observed on many types of cancer.

5.1.5. Liver cancer. The serum level of alpha-fetoprotein (AFP) is a biomarker for liver cancer but has low sensitivity. An alternative to measuring AFP alone is to measure the fraction of AFP that is fucosylated (AFP-L3), since the fucosyltransferase required for AFP-L3 synthesis is elevated in liver tumors and is believed to promote malignant transformation. AFP-L3 levels of 10% or above have been proposed as a diagnostic for cancer, and a previous study estimated that AFP-L3 levels surpass this threshold several months prior to when liver cancer becomes detectable *via* imaging.[242–244] AFP-L3 levels can be monitored *via* ELISA, but Wu *et al.* achieved more reliable results by using glycan microarrays to detect serum antibodies to AFP-L3.[245] Their array, consisting of both fucosylated and nonfucosylated AFP glycoforms, was used to screen serum samples from hepatocellular carcinoma patients (HCC, n = 32) and hepatitis B patients (n = 8). Whereas neither AFP nor AFP-L3 levels effectively discriminated HCC from non-cancerous disease when measured *via* ELISA, significantly higher anti-AFP-L3 antibody levels were measured in HCC *via* the glycan array (p = 0.014).

The specificity of screening for liver cancer can also be improved using a panel of glycan biomarkers. Seeking TACA-targeting serum antibodies to serve as improved liver cancer biomarkers, Wu *et al.* constructed a glycan array of 58 synthetic glycans and tested serum from subjects with HCC (n = 293), hepatitis B (n = 133), hepatitis C (n = 134), or no disease

(n = 33).[246] After normalizing signals relative to the anti-Gb5 IgG for each sample, cancer serum contained significantly elevated ($p < 0.05$) IgG levels against seven glycans. These glycans were sialyl LacNAc $6SO_3$, disialosyl galactosyl-globoside (DSGG), Fuc-GM1, Gb3, Gb2, B19, and Man7. DSGG, a GSL, had been studied in renal cell carcinoma,[247] small-cell lung cancer,[248] and metastatic cancer,[247] but had not previously been linked to HCC. Anti-glycan antibody signals to DSGG, Fuc-GM1, and Gb2 identified HCC cases nearly twice as accurately as AFP levels alone. Using both the antibody signals and the AFP level in tandem further increased the diagnostic sensitivity to 80% in male patients.

5.1.6. Lymphoma. To study anti-glycan antibodies in lymphoma patient serum, Lawrie *et al.* performed screening on a 37-component GlycoChip array comprised of a range of human and plant sugars, mostly monosaccharides and disaccharides.[249] Comparing patients with classical Hodgkin's lymphoma (cHL, n = 8) to age- and sex-matched controls revealed differences in antibody signals. In the cancer cohort, elevated signal was observed to five glycans: L-Ara*f*, Fuc(α), Fuc(α1-2)Gal-β, Tn, and GalNAc(β). Subsequent ELISA experiments on samples from larger cohorts of cHL patients (n = 16), diffuse large B cell lymphoma patients (n = 18), and controls (n = 12) confirmed significantly elevated levels of L-Ara*f* and GalNAc(α) antibodies in cHL relative to other lymphomas and controls. The authors hypothesize that the elevated levels of anti-Tn IgM in cHL patients were induced by elevated Tn antigen expression in this cancer subset: five of eight samples assessed using immunohistochemical staining were Tn positive.

5.1.7. Gastric cancer. Serum screening on protein microarrays has identified elevated antibodies to MUC1 in gastric cancer patients up to nine years before clinical diagnosis.[250] Although these studies largely focused on antibodies to the unglycosylated mucin protein backbone and other protein antigens, antibodies to the many glycoproteins and other glycan antigens known to be differentially expressed in gastric cancer[251] also merit investigation as serum biomarkers. A recent study from Shilova *et al.* exemplified the potential of such glycan makers.[252] Sera from gastric cancer patients (n = 146) and healthy controls (n = 55) were compared on a 300-component glycan array, which identified a signature of IgM to seven targets and IgG to four targets (including both IgM and IgG against TF) that correctly diagnosed 81% of cases. As discussed in Section 4.1, glycan microarray screening has also been used to identify distinct serum antibody binding profiles in patients with *H. pylori* infection compared to uninfected individuals.[169] *H. pylori* infection doubles the risk of gastric cancer development.[253] Since the immune response to infection is known to at least partially mediate the link between *H. pylori* and cancer,[254] antibody information gleaned from glycan microarrays may help elucidate this mechanism.

5.1.8. Pancreatic cancer. In their comprehensive 2013 study, Burford *et al.* screened sera against an array of aberrantly glycosylated mucin glycopeptides, as discussed above in the context of breast cancer.[219] When comparing pancreatic cancer patients (n = 35) and controls (n = 247), no significant differences between the cohorts' IgG against core 3-, Tn-, or sTn-modified MUC1 were identified. However, more recent immunohistochemical studies have identified sialyl Lewis X (sLeX)-MUC1 as being much more highly expressed in pancreatic cancer tissue than sTn-MUC1, so antibodies to sLeX-modified mucins or alternative mucin glycopeptides may prove to be more effective diagnostics.[255,256] It is also likely that a panel of anti-glycan antibodies may perform better than individual anti-mucin antibodies as pancreatic cancer biomarkers. A review of a large panel of antibodies to 124 different glycoprotein, peptide, and protein targets concluded that while individual serum antibodies are not effective pancreatic cancer biomarkers (average sensitivity 22%), a combination of serum antibodies dramatically improves diagnostic sensitivity (to as high as 73%).[257] The glycan microarray format is well-suited to identifying this type of diagnostic signature in serum.

Beyond screening serum, the 609-component CFG glycan array has also been used to screen IgA in gastrointestinal lavage fluid samples isolated from pancreatic cancer patients (n = 14) and controls (n = 6).[258] The authors focus on IgA because mucosal-associated lymphoid tissue in the GI tract is hypothesized to secrete IgA against pancreatic cancer glycoproteins. Interestingly, half of the antibody signals observed to be elevated in pancreatic cancer patients targeted glycans sharing a terminal GlcNAcα1-4Galβ1-4GlcNAc motif. This motif is common to the bacterial lipopolysaccharide *P. mirabilis*, which had previously been linked to inflammation but not to cancer.

5.1.9. Oral squamous cell carcinoma. Altered glycosylation, including aberrant *N*-glycosylation of glycoproteins and upregulation in sLeX expression, is well-documented in oral squamous cell carcinoma (OSCC) and other head and neck squamous cell carcinomas.[259,260] Many of these glycans have been directly linked to cancer progression, immune evasion, and metastasis.[259] While chip-based screening had previously been used to identify anti-p53 antibodies in OSCC patient saliva,[260] Guu *et al.* applied OBI-Pharma's Glycan-23 Chip to identify anti-glycan antibodies in OSCC patient serum.[261] Sera from OSCC patients (n = 65) and healthy controls (n = 21) were analyzed on the 22-component glycan microarray, and cancer patients were found to have elevated IgM to two glycans (SSEA-3 and GD2) and diminished IgG to nine glycans (including GD2, Globo H, LeY, and sLeX).

5.2. Prognosis, staging, and predictors of response to treatment

Associations between glycan expression and cancer prognosis are well documented and have been observed for decades.[262,263] These relationships are not coincidental: many of the cancer-associated glycans correlated with disease progression are directly involved in processes related to cancer metastasis, including cell adhesion, immune evasion, and cell mobility. Besides the levels of the glycan antigens themselves, levels of serum antibodies against these glycans have also been linked to cancer prognosis. Antibodies to MUC1, for example, are associated with better survival in patients with gastric,[264] lung,[265]

breast,[266] and pancreatic cancer.[267] Antibody levels to Tn and TF have also been found to correlate with survival in gastric cancer,[268] breast cancer,[269] and a range of other carcinomas.[270] These associations are not always straightforward. In some cases, higher levels of antibodies against tumor glycans correlate with improved prognosis, perhaps because they indicate a more effective anti-tumor immune response. In other cases, however, higher levels of antibodies correlate with worse prognosis. For example, high levels of serum IgG/IgM to Man9 in prostate cancer patients significantly correlate with larger, higher-grade, more aggressive disease.[271] While many of these studies have historically relied on ELISA assays to measure serum antibodies against a very limited set of glycans, using glycan arrays enables broader serum profiling against more diverse glycan targets that may correlate with patient outcomes and aid physicians in effectively tailoring treatments.

5.2.1. Anti-glycan antibodies as biomarkers for cancer stage and prognosis. In addition to evaluating anti-MUC1 serum antibodies as a diagnostic, Blixt *et al.* also evaluated these antibodies for their prognostic potential.[224] They found that IgG levels to all MUC1 glycoforms present on their 31-component array correlated with breast cancer tumor grade. Furthermore, levels of antibodies against a particular subset of core 3- and sTn-modified MUC1 glycopeptides were particularly high and significantly associated with both tumor grade (p = 0.016) and time to metastasis (p = 0.028).[224]

In addition to natural glycopeptide arrays, other MUC1 arrays include modified mucin glycopeptides that bind to serum antibodies with enhanced affinity or specificity. Building on previous work in which the peptide backbone of MUC1 was modified with proline mimics,[272] Guillen-Poza *et al.* used MUC1 peptides modified with an iminosugar GalNAc mimic in place of the natural GalNAc antigen, hypothesizing that the altered glycopeptide conformation might increase serum antibody binding.[273] In a preliminary analysis, significantly different binding profiles were observed not just between serum from healthy controls (n = 4) *versus* people with breast cancer, but also between early- *versus* late-stage cancer serum.[273]

In Tikhonov and coworkers' screening of serum from colon cancer patients (n = 44), elevated levels of IgM to 3′-*O*-sulfo-LeA were determined not only to distinguish cancer cases from controls (p = 0.03), but also to distinguish patients with (n = 18) and without (n = 26) metastases in their lymph nodes (p = 0.008). Antibodies to several other targets, including LeC, sLeC, and sLeA, were also found to be associated with both the degree of tumor differentiation and the location of the tumor.[236]

Vuskovic *et al.* screened serum from mesothelioma patients (n = 35) and determined that elevated IgG, IgM, and IgA antibody signals to two of the 211 glycans on the array (Glcα1-4Glc and Glcβ1-6Gal) were associated with increased likelihood of survival past 28 months.[93] These prognostically useful glycans were different from the best-performing diagnostic glycan, Neu5Acα2-3Galβ1-4Glc, which yielded an AUC of 0.7274 in ROC analysis when distinguishing mesothelioma patients (n = 50) from high-risk, asbestos-exposed controls (n = 65).

In Jacob and coworkers' screening of non-mucinous ovarian cancer serum on a 203-component array, low IgM to the P_1 GSL was found to not just be diagnostic for cancer cases *versus* controls, as discussed above, but also to be prognostic for survival. Anti-P_1 IgM levels were significantly associated with the stage of disease (I/II *vs.* III/IV), although not with grade or tumor origin. Low anti-P_1 IgM was also associated with more than twice the risk of earlier cancer relapse (HR 2.328, 95% CI 0.96–5.64).[231]

More recently, Purohit *et al.* analyzed serum from ovarian cancer patients (n = 119) on their multiplex glycan bead array consisting of 184 glycans conjugated to 184 Luminex beads with unique spectra.[79] Serum samples were obtained from patients prior to treatment (surgery or chemotherapy), during chemotherapy, and after remission for the 76 patients who achieved remission. Using the Luminex array to measure anti-glycan IgG in serum at the time of remission, the authors classified signals into ''high'' or ''low'' IgG levels for each glycan array component, then used this information to construct Kaplan–Meier survival curves. High IgG against P_k was significantly associated with shorter survival from the time of diagnosis to death (HR = 3.99, p = 0.00301), and high IgG against blood group A antigen was significantly associated with longer survival (HR = 0.31, p = 0.0048).[79]

Anti-glycan antibody profiling has also proven useful for prognosis in instances where it was not applicable to diagnosis. Pedersen *et al.* used a microarray of recombinant MUC1, MUC2, and MUC4 peptides to compare serum collected from patients at the time of colorectal cancer diagnosis (n = 157) to healthy controls (n = 40).[238] Although serum IgG to MUC1 glycopeptides was significantly elevated in this case-control set, these results did not hold in prospectively collected serum from the UKCTOCS databank for controls (n = 94) and women who went on to develop colorectal cancer (n = 97). Nonetheless, the array results did demonstrate prognostic value: elevated antibodies against the Tn-modified MUC4 peptide MUC4TR5 were significantly associated with increased mortality (p = 0.000011).[238]

5.2.2. Anti-glycan antibodies that predict treatment efficacy. Great progress has been made in better characterizing the molecular markers of different cancer subtypes, enabling the targeting of treatment regimens to a patient's particular tumor. One future direction for this type of personalized medicine is the use of a patient's anti-glycan antibody profile as a marker to predict the efficacy of different treatment regimens.

In one example, the Luminex glycan array was applied to analyze serum from cervical cancer patients with stage II (n = 276) or stage III (n = 292) disease.[274] Elevated antibody signals to 13 of the 177 glycan-conjugated beads used in the array were associated with better survival for patients treated with combined external beam radiation therapy and brachytherapy as opposed to external beam radiation therapy alone. The glycans with predictive value include those from the blood group H, Lewis, isoglobo, and lacto families.[274] The authors postulate that serum anti-glycan antibodies to these targets might be associated with improved survival either because these

antibodies directly neutralize glycans associated with tumor growth, or because the presence of these antibodies indicates a generally active immune system that is able to fight cancer. While more research is needed to establish the reason for this association, this study demonstrates the potential role of serum anti-glycan antibodies in treatment selection.

Pre-existing anti-glycan antibodies in serum might also predict a patient's response to vaccine therapeutics. In a clinical trial of the PROSTVAC prostate cancer vaccine, serum was taken from prostate cancer patients (n = 28 for the training set; n = 76 for the validation set) prior to vaccination with the PROSTVAC vaccine, which consists of a poxvirus vector encoding prostate-specific antigen. Screening on a 171-component glycan array determined that pre-vaccination IgM to blood group A (BG-A) trisaccharide was significantly associated with post-vaccination survival (p = 0.005 in the validation set).[275,276] For patients in the upper quartile of anti-BG-A IgM prior to vaccination, median survival was nearly double that of both patients in the lowest quartile (p = 0.01) and control subjects who did not receive the vaccine. The control subjects (n = 37) receiving empty poxvirus vector alone did not exhibit any association between anti-BG-A trisaccharide IgM and survival.

One postulated mechanism for the association between pre-vaccination anti-BG-A IgM and survival only in the PROSTVAC-vaccinated cohort is that the vaccine, produced in chicken embryos, may display BG-A-like glycans. The authors of this paper confirm the presence of these glycans in PROSTVAC and conclude that, in patients with pre-existing anti-BG-A antibodies, these circulating antibodies might bind to epitopes on the vaccine vector upon vaccination. The resulting increase in the immunogenicity of the vaccine could enhance the immune response, boosting survival.[275] Pre-existing anti-BG-A titers might therefore be a useful clinical criterion for deciding which patients should be prescribed PROSTVAC, and perhaps other poxvirus-based cancer vaccines as well.

5.3. Antibody response to cancer vaccines

Glycan microarray screening can provide valuable information about the immune response to a vaccine throughout the process of vaccine development, potentially helping with injection schedule optimization, adjuvant selection, and construction of the immunizing antigen. Here, we overview the applications of serum profiling on glycan microarrays to three aspects of glycan-based vaccines: (1) engineering improved vaccines, (2) assessing vaccine efficacy in clinical trials, and (3) understand the mechanisms of vaccine-induced anti-cancer activity.

5.3.1. Vaccine constructs. Vaccines generating anti-glycan antibodies are promising targeted cancer therapies; however, successfully inducing an effective and specific anti-glycan response has proven challenging. This is largely because glycan antigens elicit only a T cell-independent response: B cell class-switching and generation of a better immune response requires activation of helper T cells by immunogenic carrier proteins. Over the last several decades, significant progress has been made in engineering glycan vaccines that elicit a stronger and more specific antibody response. Some of these strategies include employing alternative carrier proteins or conjugation chemistries to tailor the immune response, synthesizing modified glycans to enhance immunogenicity, and using glycopeptide-based vaccines to target specific cancer-associated mucins.

Carrier proteins, linkers, and antigen density in vaccine design. While proteins like diphtheria toxin and keyhole limpet hemocyanin (KLH) are widely used as vaccine carriers, including for carbohydrate-based vaccines, achieving control over the display of antigens on these carrier proteins is difficult, and results have been mixed. Virus-like particles (VLPs)—such as tobacco mosaic virus (TMV) and Qβ bacteriophage (Qβ)—are alternative carriers. These constructs offer the opportunity to engineer viral coat proteins to display antigens in a repetitive and organized manner, improving B cell receptor clustering and response.

Huang and coworkers used a copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction to conjugate the Tn antigen to VLPs, creating several different Tn vaccines.[277] In the first vaccine construct, a Tn antigen-bearing amino acid was conjugated to TMV coat proteins.[277] Conjugation of the Tn-amino acid to an inserted cysteine residue at the TMV coat protein's *N*-terminus did not prove effective. However, use of Tyr139 as an alternative site for Tn functionalization produced a vaccine with more than 2000 Tn antigens per TMV capsid. In a later iteration of the Tn vaccine, over 300 copies of Tn were attached to surface-exposed lysines on the Qβ VLP.[278] Both the TMV- and Qβ-based vaccines induced a significant antibody response in mice and elicited IgG as well as IgM against Tn, indicating that the VLP platforms enable isotype switching *via* helper T cell activation.

Screening of sera from mice vaccinated with either the TMV- or the Qβ-based Tn vaccine on a 329-component carbohydrate microarray consisting of glycopeptides, glycoproteins, and BSA-linked glycans demonstrated the specificity of the immune response. In both the TMV- and the Qβ-based treatment groups, serum antibody binding was specific for Tn glycopeptides and glycans with a terminal GalNAc, with no binding to unglycosylated peptides or glycan epitopes with an internal GalNAc. The glycan array used for these studies also included neoglycoproteins displaying the same glycan at different densities. Leveraging this feature, the authors concluded that the antibody response from the Qβ-based vaccine was stronger against higher density glycans. They also noted that a higher dose of the vaccine elicited a more specific response.[278]

Sera from both TMV- and Qβ-vaccinated mice bound to the Tn-positive Jurkat cell line, derived from a T-cell leukemia patient. However, subsequent experiments by Yin *et al.* determined that the sera from mice receiving the Qβ-based vaccine were not reactive against a second Tn-positive cell line, the aggressive murine mammary cancer TA3Ha.[279] This was at least in part due to the rapid generation of antibodies binding the triazole motif formed by the CuAAC reaction used to construct

the vaccines, blocking access of B cell receptors to the glycan antigen.

When Tn was conjugated to the Qβ VLP scaffold *via* NHS ester instead of CuAAC chemistry, the resulting vaccine construct elicited four times higher titers of anti-Tn IgG in mice. Sera from mice immunized with this NHS-conjugated vaccine compared to the CuAAC-conjugated vaccine construct demonstrated improved binding to Tn-expressing cancer cell lines ($p < 0.01$) and broader binding on the glycan microarray. This enhanced binding profile included binding to Tn-threonine, even though the vaccine itself only included Tn-serine. Serum antibody binding was observed to both Tn monomers (Tn-serine and Tn-threonine) and clusters (Tn-serine only), and sera from mice that survived cancer challenge post-vaccination were found to have more binding to Tn-serine peptides than sera from mice that succumbed following post-vaccination cancer challenge ($p = 0.03$).[279]

The favorable outcomes for mice that generated antibody responses against diverse Tn targets provide *in vivo* evidence for the importance of inducing a diverse antibody response *via* vaccination. Because TACAs may be expressed on a broad spectrum of protein and lipid carriers, vaccine-induced antibodies must target their cognate glycans in many different contexts to achieve an effective anti-cancer response. The breadth of the antibody response, not just the magnitude, is critical for clinical success.

Beyond Tn, other glycan motifs such as *N*-linked glycans are a desirable but challenging target for vaccines against HIV and cancer. To assess potential *N*-linked glycan vaccine constructs, Donahue *et al.* immunized mice with a set of five representative *N*-glycans conjugated to Qβ VLP.[280] The immunization elicited high IgG and low IgM titers, indicating that successful class switching was achieved. However, the authors found that the induced antibody titers depended on the linker used to conjugate the glycans to Qβ. Higher titers and stronger binding were observed in mice vaccinated with the adipic acid-conjugated antigen compared to the CuAAC-conjugated antigen, again suggesting that the immunogenicity of the CuAAC triazole itself can inhibit the anti-glycan response. Additionally, despite observing signal to the immunizing glycans *via* ELISA, the authors found no binding to the expected serum motifs when screening sera *via* an 816-component glycan microarray. This was apparently due to the format of the glycoconjugates on the array, as an alternative glycan array with intact *N*-linked glycans (as opposed to a ring-opened first GlcNAc residue) showed the expected binding. This binding profile on the microarrays suggested that much of the *N*-linked-glycan reactivity generated by the vaccines is directed against the innermost chitobiose core, which was confirmed by further investigation of the target epitopes using ELISA. Because this chitobiose motif is so immunodominant, generating antibodies against more specific, extended *N*-glycan motifs remains a challenge for the TACA vaccine field.

Most recently, the Qβ-scaffold format has been applied for immunization of mice with sLeA. This vaccine format produced much higher titers of anti-sLeA IgG than an earlier sLeA vaccine with a KLH carrier that advanced to clinical trials.[281] Microarray analysis of sera from the immunized mice demonstrated that the induced IgG bound selectively to sLeA but not to most other related Lewis antigens.[282] In addition to the identity of the carrier and the linker, the density at which the carbohydrate antigen is presented can be important. The Gildersleeve group investigated this factor by preparing a Tn-modified tripeptide (mimicking a sequence found in many mucin tandem repeat regions) conjugated to human serum albumin (HSA) at low or high density.[283] Rabbits were then vaccinated with either the low- or high-density HSA conjugate. Although the overall magnitude of the IgG response was similar for both groups of rabbits, profiling on a 170-component glycan array consisting of neoglycoproteins and native glycoproteins yielded information about how the antibody response was affected by the density of the immunogen. High density was found to induce broader reactivity against Tn antigens in diverse peptide sequences, Tn linked to both serine and threonine, and Tn displayed as both single and clustered antigens.

Modified glycans as immunogens. The use of modified, more immunogenic glycans is another vaccine design strategy to elicit a stronger, more specific anti-TACA antibody response. Various types of modified glycan antigens—including ganglioside lactones, non-native glycoside linkages, and fluorinated glycans—have been investigated as vaccines, as reviewed by Yin and Huang.[284] For immunization with modified glycans to succeed in the clinic, the elicited antibody response must target not just the synthetic immunogen, but also the naturally occurring TACA target. Glycan microarray profiling of vaccinated subjects' sera is useful in assessing whether this cross-reactivity is achieved. For example, Sahabuddin *et al.* used a small, sTn-based microarray to demonstrate that their *N*-propionyl-modified sTn vaccine was not just immunogenic, but also able to produce a response against natural sTn.[285]

In most cases, modifications to glycans in TACA vaccines have been tested on a trial-and-error basis. There is a demand for a more comprehensive method to assess the effects of synthetic substitutions on the immune response. Lee *et al.* systematically modified the Globo H antigen at the C6 position of either the reducing-end glucose or nonreducing-end fucose, producing 10 Globo H analogs with various unnatural substitutions.[286] These glycans were then conjugated to a diphtheria toxin carrier and administered with a C34 glycolipid adjuvant, a vaccine construct known to effectively elicit an anti-Globo H IgG response, as assessed by glycan microarray.[287] The authors tested sera from mice immunized with the Globo H vaccines on a 94-component glycan microarray consisting of TACAs, Globo H, modified Globo H analogs, and various linkers.

Their screening confirmed that the synthetic Globo H analog vaccines elicited a strong IgG response. Although the induced antibodies bound most strongly to the modified Globo H targets, they also exhibited binding to natural Globo H. In fact, the antibodies induced by the modified Globo H analogs bound unmodified Globo H significantly better than the

antibodies induced by an unmodified Globo H vaccine. Specifically, azido modification at the nonreducing end or azido/fluoro/phenyl modifications at the reducing end generated a strong IgG response to Globo H and the related epitopes Gb5 and SSEA-4. This IgG response is reactive against Globo H-positive MCF7 cells and was able to trigger complement-dependent cytotoxicity *in vitro*. More recently, additional Globo H analog vaccines were tested in mice. These vaccines contained azido modifications of the internal sugars. As assessed by profiling of serum from vaccinated mice on a glycan microarray, azido-modification of C6 of the galactose closest to the reducing end produced the most effective vaccine.[288]

In another example of synthetic glycan vaccines, GD2 analogs have been used in attempts to induce a stronger, more cancer-specific immune response. The GD2 ganglioside is overexpressed on cancer cells, but it is also found on healthy peripheral nerve fibers; thus, immunotherapies targeting GD2 often lead to debilitating pain. Targeting 9-*O*-acetylated GD2 (9OAc-GD2), rather than GD2, may reduce this side effect. Unlike GD2, 9Oac-GD2 is found on cancer cells but not on nerve fibers. While 9OAc-GD2 itself is too unstable to be used for vaccines, Wu *et al.* demonstrated that a Qβ-based vaccine of 9NHAc-GD2—an analog of 9OAc-GD2 that is less susceptible to hydrolysis—could induce a strong and specific IgG response against acetylated GD2.[289] Screening of serum from mice vaccinated with both natural GD2 and 9NHAc-GD2 on a 738-component carbohydrate microarray demonstrated that natural GD2 elicited a weaker and broader response against GD2, acetylated GD2, and other gangliosides. In contrast, 9NHAc-GD2 elicited a stronger and more selective response against only acetylated GD2. When tested *in vitro*, sera from 9NHAc-GD2-immunized mice also bound and killed cancer cells better than sera from mice immunized with natural GD2.

Glycopeptide-based vaccines. The same glycan antigen may be displayed on a wide range of glycoproteins or glycolipids on cancer cells. The goal of carbohydrate-based vaccines is to generate an immune response to the cognate glycan when it is presented in any of these contexts. However, glycopeptide vaccines may be used to target a glycan modification in the context of one specific protein. This approach has been especially popular with cancer-associated mucins.

The specificity of the immune response to vaccination with mucin glycopeptides has been demonstrated in several ways, including by Steentoft *et al.*, who attempted to specifically define the immunodominant epitope of a murine mucin glycoprotein, podoplanin. The authors synthesized a Tn glycopeptide covering a 30-mer region of the podoplanin protein, then conjugated it to a KLH carrier and used this antigen to immunize mice. Analyzing the sera from the mice using a glycopeptide microarray, they found no reactivity to unglycosylated peptide or to Tn on an irrelevant peptide, but high IgG levels to the glycopeptide of interest. This is evidence of a highly specific antibody response to the combined glycopeptide motif, not to the glycan or the peptide itself.[290]

Tn-MUC1 glycopeptide vaccines have also been tested in cancer patients. In these studies, glycan microarray analysis has demonstrated that the human immune response remains highly specific for the glycopeptide motif, as opposed to the unglycosylated peptide or the glycan in other contexts. Nonetheless, there is significant variability in the antibodies generated by vaccination. The exact binding profile observed when screening serum on microarrays depends greatly both on the exact mucin glycopeptide used for vaccination and on the individual patient.[218,291,292]

5.3.2. Patient responses in vaccine clinical trials. Glycan microarrays have proven useful for analyzing cancer patients' immune response to vaccines in clinical trials. In a 2010 study, sera from six patients were analyzed both pre-immunization and post-immunization with the PROSTVAC cancer vaccine.[103] Although the main target of the vaccine is prostate-specific antigen (PSA), there are several ways the vaccine might induce anti-glycan antibody response. PSA is a glycoprotein, and the phenomenon of antigen spreading could lead to responses to abnormal glycosylation found on prostate tumors. The array used in this study consists of 147 neoglycoproteins and glycoproteins mixed with BSA at four different concentrations, ranging from 0% to 87.5% unconjugated BSA. Following vaccination, levels of antibodies to Forssman antigen increased, and the increase in binding was significantly affected by the neoglycoprotein density.

In a subsequent study, a glycan microarray with 171 structurally distinct glycans was used to profile sera from prostate cancer patients (n = 28) pre- and post-PROSTVAC vaccination.[68] Although the overall antibody profile did not change dramatically, increased responses to particular glycans were observed. One such glycan was the Forssman xenoantigen: antibodies to this target increased in 64% of patients in the discovery set, but not in healthy controls or nonvaccinated prostate cancer patients. Overall survival positively correlated with increased anti-Forssman antigen antibody levels, and this was confirmed in a validation cohort of 76 vaccine recipients and 37 controls who received the vaccine viral vector, but not the full vaccine.[293] Patients vaccinated with the control vector had similar antibody responses to the Forssman antigen, but these antibody responses did not correlate with survival, suggesting that the anti-Forssman antigen antibody response is directed at the viral vector itself. This is likely because chicken embryo dermal cells, which express these glycan antigens, were used as host cells for vaccine production. The authors propose that these glycan xenoantigens are acting as an adjuvant and boosting the immune response to PROSTVAC treatment.

The GVAX whole cell vaccine, which targets metastatic pancreatic cancer, is also in clinical trials. In an effort to understand the anti-glycan immune response triggered by vaccination, Xia *et al.* profiled serum from 28 pancreatic cancer patients enrolled in the clinical trial.[294] IgG and IgM levels were measured on a 407-component glycan microarray at three different timepoints: prior to vaccination, after one vaccine dose, and after three vaccine doses. Although pre-vaccination pancreatic patient serum showed a similar profile to that of

healthy controls, vaccination elicited a significant increase in antibody signals to certain glycans, including many TACAs. There was an increase in IgG against sLeX, sTn, and the Neu5Gc variant of GM3, and an increase in IgM against LeY. Significant responses were also observed to nonhuman glycans and glycoproteins, particularly bovine fetuin and α-Gal. The authors hypothesize that these antibodies were induced by non-human glycans incorporated during vaccine production, as pre-incubation of sera with fetal bovine serum (FBS) significantly abrogated array binding to fetuin and α-Gal.

Importantly, antibody signals to various α-Gal array components inversely correlated with survival: the median survival time for patients with high levels of anti-α-Gal IgG was much shorter than for patients with low anti-α-Gal IgG ($p = 0.003$). The authors hypothesize that a strong immune response to non-human targets outcompetes the immune response to vaccine tumor antigens, impairing vaccine efficacy and reducing survival. Evidence for this explanation is that antibodies to galectin-3, previously demonstrated to correlate with survival, are primarily observed in patients without an α-Gal response. These anti-non-human glycan responses were also identified in serum of patients receiving other whole-cell cancer vaccines grown in HSA-supplemented media, but not in patients receiving vaccines grown in serum-free media.[294] The results suggested that strategies to minimize the presence of FBS in whole cell vaccine preparations might improve their efficacy. Based on the results, a more extensive wash protocol is now being used to prepare the GVAX-H1299 whole cell vaccine for clinical trials, illustrating how glycan microarray profiling can influence clinical development.

5.3.3. Response to other cancer therapies. Like cancer vaccines, a variety of other cancer therapies can trigger anti-glycan immune reactions. These antibody responses are often an unintended result of ''non-self'' glycosylation patterns found in therapeutic agents produced in plants or animals. For example, rabbit anti-human thymocyte globulin (ATG) is an immunosuppressant derived from rabbit serum occasionally used in combination with other cancer therapies. ATG has been found to induce an anti-Neu5Gc antibody response, as studied *via* glycan microarrays.[295] Similarly, anti-arabinose antibodies found in lymphoma patient serum have been postulated to be generated in response to the chemotherapeutic agent cytosine arabinoside.[249] The immunotherapy cetuximab, produced in a murine cell line, has triggered dangerous IgE allergic responses directed against its non-human α-Gal glycosylation.[296]

In other cases, however, unintended anti-glycan antibody responses to therapeutics have beneficial anti-cancer activity. Glycan arrays have proven extremely useful in probing the anti-glycan antibody repertoire following administration of these therapeutics. This information has bolstered our understanding of therapeutics' mechanisms of action and offered insight into the role of anti-glycan antibodies in fighting cancer. Two examples of such therapies, KLH and Reishi mushrooms, are discussed here.

As described earlier in the context of vaccination, KLH is often used as a carrier protein for other peptide and glycan vaccines due to its high immunogenicity. However, KLH alone has demonstrated potent anti-cancer activity and has been approved as a treatment to prevent bladder cancer recurrence after surgery. The mechanism by which KLH fights cancer is unclear, but it has been hypothesized to stimulate the immune system, possibly by inducing tumor-binding antibodies. To test this hypothesis, Oyelaran *et al.* used a 107-component microarray of glycoconjugates and glycoproteins to profile antibodies induced by KLH treatment.[297] Serum samples from 14 individuals were profiled before and after immunization with KLH and an alum adjuvant. Follow vaccination, anti-KLH antibody levels increased, especially those targeting carbohydrate epitopes on KLH. There were also changes in antibody levels to other carbohydrate epitopes, including the TACAs LeY, dimeric LeX, and sLeA. Of note, pre-vaccination antibody levels to six antigens (including KLH itself, as well as GlcNAc1-4Gal and lactoseries tetrasaccharide c) were shown to have a statistically significant inverse correlation with KLH response. This suggests that preexisting antibodies to these antigens may serve as biomarkers for bladder cancer patients unlikely to respond well to KLH treatment.[297]

Microarray profiling has also revealed that anti-glycan antibodies mediate the anti-cancer activity of Reishi mushrooms. Long used as an herbal medicine, Reishi mushrooms (*Ganoderma lucidum*) contain fucose-containing polysaccharides (F3) that are known to activate the immune response against cancer. Liao *et al.* demonstrated that this antitumor immunity is antibody-mediated.[298] Glycan microarray screening of F3-induced antisera in mice was carried out to assess binding to 60 synthetic oligosaccharides, including several TACAs. The authors observed increased binding of IgM to Globo H series antigens in F3-treated mice compared to the control group.

Liao *et al.* also produced a fucose-enriched F3 fraction (FMS). Mice were injected with this FMS fraction, the original unenriched F3 fraction, or PBS control; serum was then analyzed on the 611-component CFG glycan microarray to assess IgM antibody response. Compared to the antibody response from F3-treated mice, the antibodies from FMS-treated mice showed higher specificity and selectivity for many of the array components. Glycan components that gave the strongest signals on the CFG array were classified into two families of TACAs: the blood group ABO(H) antigens and the globo-series glycans. Suspecting that the terminal fucose on the glycans present in FMS drives anti-tumor immunity, the authors used a fucosidase to remove 50% of the fucose in FMS. The original FMS fraction and the defucosylated fraction were then evaluated for the treatment of mice subjected to cancer challenge. The original FMS fraction significantly reduced lung tumor volume and inflammatory chemokine levels in treated mice compared to controls. Defucosylated FMS, however, failed to produce the same results. Mice injected with the defucoyslated-FMS also demonstrated reduced serum IgM binding to Globo H, as detected by the glycan microarray.[298]

6. Autoimmune diseases, other diseases, and other applications

Glycan arrays have been used to profile serum antibodies in a variety of other diseases and conditions, such as autoimmune diseases and organ transplants. These studies are summarized in Table 4 and discussed further in this section.

6.1. Allergy and asthma

As discussed in the context of schistosome infection in Section 4.3.1, exposure to helminths and other parasites can induce an anti-glycan IgE response. These antibodies may cross-react with environmental or food allergens but do not typically produce allergy symptoms. To elucidate specific glycan motifs associated with this condition, Amoah *et al.* characterized IgE responses in children from helminth-endemic areas in rural Ghana by screening sera on microarrays printed with 126 synthetic *N*-glycans.[182] Cross-reactive IgE in Ghanaian children was observed to bind both core α1-3 fucose and core xylose motifs.

In a separate study in Uganda, Nkurunungi *et al.* studied IgE responses in a large set of subjects from rural *Schistosoma mansoni*-endemic communities (n = 208), proximal urban communities (n = 62), and from an asthma case-control study (50 cases and 50 controls).[299] These plasma samples were profiled on an array with 135 chemo-enzymatically prepared *N*-glycans, with and without β1-2-xylosylation and/or α1-3-fucosylation. Over 40% of the subjects had serum antibodies to carbohydrate-related allergens. Reactivity to certain core α1-3-fucosylated *N*-glycans was inversely correlated with asthma.

Both allergic and non-allergic bronchial asthma affect many children and adults worldwide. In their 2018 study, Butvilovskaya *et al.* screened serum from children diagnosed with bronchial asthma, representing the first time that the anti-glycan antibody repertoire has been profiled in this patient population.[300] The authors use a 3D hydrogel array format in which gel microdrops were printed on a slide, antigen array components were added, and spots were polymerized under UV light. In addition to 55 glycan antigens, the array also included anti-IgG, -IgA, and -IgM antibodies to quantify the amounts of each isotype in serum. Comparing serum profiles from healthy 4- to 17-year-old patients (n = 60) and those with exacerbated bronchial asthma (n = 35), the levels of total IgM between the two cohorts did not significantly differ. However, the authors observed systemic decreases in IgM against specific glycans in the bronchial asthma patients. Broadly, this included many sialylated and sulfated glycan derivatives, *O*-linked glycans, and glycans containing LacNAc, GlcNac, Man, and Gal residues. Butvilovskaya *et al.* propose that these lower levels of IgM may be related to phenomena such as damage-associated molecular pattern pathways in eosinophilic inflammation or the binding of some bronchial asthma-associated allergens to other immune receptors instead of IgM.

Table 4 Summary of glycan microarrays tested with human serum from diseased status and other abnormal conditions[a]

Condition or disease	# of glycans	Glycan types	# of samples screened	Serum dilution	Array format	Isotype	Ref.
Allergy	126	*N*-Glycans and short oligosaccharides	69	1 : 30	NHS-Functionalized glass slide	IgE	182
Nickel exposure	380	Synthetic glycans	89	1 : 15	NHS-Functionalized glass slide	Combined IgG/A/M	301
Guillain–Barré	25	Synthetic glycans	17 (12 + 5)	1 : 500	NHS-Functionalized glass slide	IgG, IgM	140
Diabetes	202	Synthetic glycans	688	1 : 500	Suspension array	IgG	308
SARS-CoV-2	816	Glycoproteins, neoglycoproteins	148 (40 + 70 + 38)	1 : 50	Epoxide-Functionalized glass slide	IgG, IgM	208
Primary antibody deficiencies	610, 147	CFG v5.1, NCFG v2, bead array	163 (76 + 5 + 25 + 6 + 8 + 43)	180 μg ml^{-1} of purified IgG	NHS-Functionalized glass slide/Suspension array	IgG	310
Kawasaki disease	48	Neu5Ac/Neu5Gc-sialoglycans	22 (6 + 6 + 10)	1 : 100	Epoxide-functionalized glass slide	IgG	311
Xenotransplant	200	Glycoproteins and synthetic glycans	7	1 : 25	NHS-Functionalized glass slide	Combined IgG/A/M	324
Burn patients	48	Neu5Ac/Neu5Gc-sialoglycans	24 (10 + 14)	1 : 250	Epoxide-functionalized glass slide	IgG	326
ATG-treated T1D	77	Neu5Ac/Neu5Gc-sialoglycans	7, affinity purified anti-Neu5Gc IgG	1 : 100, 1 μg/well	Epoxide-functionalized glass slide	IgG	295
ATG-treated kidney allograft recipients	82	Neu5Ac/Neu5Gc-sialoglycans	16	1 : 100	Epoxide-functionalized glass slide	IgG	134,328
ATG-treated kidney graft recipients after nonrenal organ transplantation	31	Neu5Ac/Neu5Gc-sialoglycans	25	1 : 100	Epoxide-functionalized glass slide	IgG	329
High red meat diet	65	Neu5Ac/Neu5Gc-sialoglycans	120	1 : 100	Epoxide-functionalized glass slide	IgG	239
Heart valve disease, bioprosthetic heart valves	64	Neu5Ac/Neu5Gc-sialoglycans	83 (59 + 8 + 16)	1 : 100	Epoxide-functionalized glass slide	IgG	316

[a] *N*-Hydroxysuccinimide (NHS); Consortium for Functional Glycomics (CFG); National Center for Functional Glycomics (NCFG); Epstein–Barr virus (EBV); rabbit anti-human thymocyte globulin (ATG).

6.2. Autoimmune neurological conditions

6.2.1. Guillain–Barré syndrome. Guillain–Barré syndrome (GBS) is a rare neurological disorder characterized by autoimmune responses against the peripheral nervous system. It is most commonly caused by a *Campylobacter jejuni* infection that induces antibodies to its lipo-oligosaccharide. These antibodies cross-react with gangliosides on peripheral nerve cells, causing polyneuropathy. Li *et al.* examined sera from GBS patients using glycan microarrays containing a large panel of glycans of the inner core of *C. jejuni* lipo-oligosaccharide extended by various ganglioside mimics, as well as their corresponding ganglio-oligosaccharides.[140] Sera from GBS patients showed strong binding to the ganglioside mimics and to GM1a. The array was able to detect anti-GM1a antibodies with better sensitivity than the traditional ELISA format used in clinical anti-GM1 assays.[140] In addition, the array enabled simultaneous detection of other ganglioside-binding antibodies, many of which cannot be evaluated in standard clinical assays. This study represents a prime example of how glycan microarrays can improve clinical information in a high-throughput fashion while using minute amounts of sera and reagents.

6.2.2. Huntington's disease. Although genetic testing can determine whether a person carries the autosomal-dominant mutation to the Huntingtin protein that invariably leads to the development of Huntington's disease (HD), genetic testing alone cannot predict when symptoms will onset. Existing tools to predict HD onset—including clinical assessment and neuroimaging—are inadequate, and better clinical biomarkers are needed to allow for therapeutic intervention prior to the onset of symptoms. Changes in brain gangliosides are well-documented in neurodegenerative diseases, including in HD patients and mouse models of HD. Moreover, immune dysregulation and activation can be measured by elevated plasma interleukins and has been documented in HD, including before symptom onset. Therefore, Lin *et al.* hypothesized that the immune response to altered gangliosides could be a potential biomarker for HD, and that a ganglioside microarray might detect these anti-ganglioside antibodies circulating in the plasma of HD patients.[302]

The authors used an array of 28 synthetic ganglioside and other glycans to screen plasma samples from healthy controls (n = 42), pre-HD patients who had not yet developed symptoms (n = 16), and HD patients (n = 39). They observed elevated anti-glycan IgM levels in pre-HD patients relative to both healthy and HD cases, but overall IgM levels did not differ. Signals to all glycans positively correlated with each other, but there was no correlation with disease burden score, age at the time of symptom onset, disease duration, or cognitive score. Additionally, only a weak correlation between antibodies targeting SLacNAc6 SO3 and Neu5Acα1-3Galβ1-3GalNAcβ1-3Galβ and years to onset was observed. As a diagnostic test for distinguishing pre-HD and HD patients, the combination of age, Huntingtin protein mutation repeat number, and anti-GD1b IgM yielded an impressive AUC of 0.95.

6.2.3. Multiple sclerosis and systemic sclerosis. Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system involving demyelination and immune attack on Schwann cells. A challenge in the treatment of MS patients is the paucity of MS-specific biomarkers for disease diagnosis and monitoring. To address these issues, various groups have leveraged glycan microarrays for the identification of MS-specific biomarkers. For example, Schwarz *et al.* used a 76-component GlycoChip array to screen sera collected prospectively from individuals who were later diagnosed with relapsing-remitting MS (RRMS, n = 107) and patients with other neurological disorders (n = 50).[303] There was a clear distinction between the groups, with anti-Glcα1-4Glcα IgM antibody levels significantly elevated in the RRMS cohort ($p < 0.0001$). This indicates a potential role for anti-Glcα1-4Glcα as a biomarker in the diagnosis of MS. In another study, cerebrospinal fluid (CSF) from 11 MS patients was profiled on a 32 carbohydrate-antigen array and compared to 9 patients with other neurological diseases.[304] In this study, IgM to clustered Man9 epitopes were significantly higher in MS patients.

Other studies have focused on the anti-glycan IgG repertoire of MS patients. Boligan *et al.* recently compared sera and CSF from RRMS patients who had not undergone previous treatment (n = 25 in both the discovery and validation cohorts) to control patients with either noninflammatory (n = 30) or non-MS inflammatory neurologic diseases (n = 22).[305] Using a 600-component CFG array along with a bead-based suspension glycan array, they determined that MS patient sera contained higher levels of IgG targeting both the xenogenic glycan Neu5Gc and the self-glycan Neu5Ac. These anti-Neu5Gc IgG antibodies may hold promise as biomarkers for MS diagnosis and therapeutic development.

Beyond MS, the repertoire of serum glycan-binding antibodies has also been explored in cases of systemic sclerosis (SSc).[306] SSc is characterized by collagen overproduction, which can cause damage to the skin and internal organs, in addition to the central nervous system. Grader-Beck *et al.* first examined SSc sera on two CFG microarrays with 320 and 377 glycan components. In an initial screening of pooled sera from SSc patients (n = 40) compared to serum from healthy donors (n = 40), ten glycans with highly elevated antibody binding signals were identified as potential biomarkers for SSc. Of these ten glycans, two were of particular interest: 4-sulfo-LacNAc and 4,6-sulfo-LacNAc, which share high structural similarity. Although levels of antibodies to these glycans were also elevated in systemic lupus erythematosus patients (n = 40), they were still several fold higher in SSc. The authors further investigated antibodies to these targets using ELISA, concluding that sulfation plays an important role in SSc immunity and that 4-sulfo-LacNAc may be a valuable SSc biomarker and therapeutic target.

6.2.4. Anti-myelin-associated glycoprotein neuropathy. Anti-myelin-associated glycoprotein (MAG) neuropathy is a rare autoimmune disease caused by a family of IgM autoantibodies that bind to myelin. Beyond pain, the presence of these autoantibodies can also result in progressive demyelination. The various target epitopes of these autoantibodies have not been fully defined, and a better understanding of their binding preferences could aid in the development of improved diagnostics and therapeutics for this condition.

One antigen of interest is the human natural killer-1 (HNK-1) trisaccharide, a carbohydrate that includes a 3-*O*-sulfated GlcA residue conjugated to a LacNAc residue; from this base, HNK-1 can be extended or modified. The HNK-1 trisaccharide is frequently found in the *N*-glycans that coat myelin-associated glycoproteins (MAGs), and IgM to these *N*-glycans may arise and cause neuropathy. These pathogenic MAG-targeting autoantibodies hold promise as a predictive and prognostic tool for patients experiencing anti-MAG-associated neuropathy. Seeking to characterize these autoantibodies' binding profiles, Bunyatov *et al.*[307] synthesized a series of six HNK-1-containing glycans and eight control glycans, which were printed into a 14-component microarray and used to profile the serum of three healthy controls and 10 patients experiencing anti-MAG neuropathy. As expected, healthy sera did not elicit any significant binding and all patient sera yielded binding only to GlcA-containing array components. More specifically, the authors note that the presence of the 3-*O*-sulfated GlcA (the HNK-1 motif) is required for optimal binding: HNK-1 glycans elicited stronger IgM signals than the glycans not containing the HNK-1 epitope. Many of the samples screened also demonstrated significant preference for the HNK-1 epitope on a lacto-neohexose backbone with or without sulfation. Because these antigens—HNK-1-containing extended LacNAc backbones—are commonly found in glycosphingolipids, Bunyatov *et al.* propose that this class of compounds may be a main target for neuropathy-causing anti-MAG antibodies.

6.3. Type 1 diabetes

Tran *et al.* used a fluorescent bead-based suspension glycan array to profile anti-glycan IgG in type 1 diabetes (T1D).[308] Analysis revealed that antibodies against aminoglycosides, blood group A and B antigens, glycolipids, ganglio-series glycans, and *O*-linked glycans were associated with islet immunity and with progression to T1D. Incorporating these anti-glycan antibodies into a diagnostic test yielded better results than clinical variables at distinguishing those with and without T1D.

Additionally, as mentioned in Section 5.3.3, alterations in the anti-glycan antibody repertoire have been observed in clinical trials of T1D patients treated with the ATG immunosuppressant to preserve beta cell function. Amon *et al.* used a glycan microarray of Neu5Gc and Neu5Ac sialoglycans to profile patient sera (n = 7) binding profiles before and after ATG treatment.[295] Increases in the levels of pre-existing anti-Neu5Gc antibodies and the generation of new anti-Neu5Gc antibodies were observed.[295] A second study from Salama *et al.* similarly observed increased global anti-α-Gal and anti-Neu5Gc IgM and IgG in T1D patients following ATG treatment.[309] Whether these anti-α-Gal and anti-Neu5Gc antibodies could unintentionally mediate inflammation and autoimmunity in T1D or transplant patients treated with ATG remains under investigation.

6.4. Primary antibody deficiencies

People with primary antibody deficiencies (PAD) often suffer from recurrent infections, autoimmune disorders, and cancer. Patients are diagnosed based on the patient's serum binding profile against a small panel of antigens. Searching for a better antibody signature for PAD profiling, Jandus *et al.* used glycan microarrays to screen 162 patients with various PAD conditions.[310] Samples included healthy controls and patients with symptomatic hypogammaglobulinemia, specific antibody deficiency, common variable immunodeficiency (CVID), CVID with low pneumococcal polysaccharide vaccination response, and IgG subclass deficiency. Serum IgG from these individuals was purified and profiled on several glycan microarrays. Although these different disorders have vastly different underlying causes, patient IgG in all PAD conditions was commonly characterized by loss of reactivity against both α-Gal and GalNAc, as well as disease-specific recognition of microbial antigens, self-antigens, and TACAs. These shared deviations in antibody profile pointed towards underlying failures of the immune system to generate anti-microbial antibodies. Overall, this analysis suggested that IgG profiling *via* glycan microarray could help precisely characterize the nature of the immune system failures in PAD.[310]

6.5. Kawasaki disease

Kawasaki disease (KD) is an acute childhood disease characterized by coronary artery inflammation that can result in thrombosis and myocardial infarction. While KD is currently diagnosed based on clinical symptoms, Padler-Karavani *et al.* hypothesized that measuring anti-Neu5Gc antibodies could assist in monitoring disease severity or progression. To assess the associations between various anti-glycan antibodies and KD, the authors used glycan microarrays of 48 sialoglycans to examine IgG of KD patients and healthy controls,[311] including patients with varying levels of coronary artery damage. Array profiling revealed that KD patients with normal coronary arteries exhibited elevated IgG binding to Neu5Gc-containing glycans (but not Neu5Ac) compared to both patients with damaged coronaries and controls. A simple assay to measure serum anti-Neu5Gc IgG and IgA responses has now been designed, facilitating analysis of these antibodies in KD or other conditions.[311]

6.6. Crohn's disease and IBD

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disease causing severe abdominal pain. IBD can be subdivided into two classes: ulcerative colitis (UC) and Crohn's disease (CD). Etiology of both UC and CD is poorly understood, and diagnostically distinguishing between the two can be challenging. In fact, indistinguishable cases are designated as a third class of IBD, termed intermediate colitis. Previous work has made use of glycan microarrays to screen IBD patients with the aim of identifying CD-specific biomarkers. Using a 34-component array in the GlycoChip format, Dotan *et al.* identified antibodies targeting laminaribioside, mannobioside, and chitobioside as discriminating biomarkers.[312,313] The presence of one of the three antibodies diagnosed CD with a sensitivity of 77.4% and a specificity of 90.6%. When at least two of the

antibodies were present, CD diagnostic specificity increased to 99.1%.[312]

In a retrospective serum screening study of one cohort of CD patients (n = 124), anti-laminaribioside and anti-mannan antibodies significantly correlated with CD-associated complications, such as stricturing, fistulizing, and additional small intestinal disease.[312] A second retrospective screening of another CD cohort (n = 755) found that antibodies to chitobioside and mannobioside were each associated with a future need for surgery ($p < 0.01$ each). Of the cohort, only 86 patients (11.3%) were negative for all anti-glycan Abs tested—the remaining 88.6% of the cohort were positive for at least one of the antibodies.[313]

The work of Pedersen *et al.* on colorectal cancer, previously discussed in Section 5.1.3, also investigated IBD.[237] Alongside sera from healthy controls and colorectal cancer patients, sera from IBD patients were screened on an array of *O*-glycopeptide components derived from human mucins (MUC1, MUC2, MUC4, MUC5AC, MUC6, and MUC7), many of which have been correlated with colorectal cancer. While many of the examined glycan epitopes, such as Tn- and sTn-MUC1, seemed to elicit antibodies only in cancer cases, core 3-MUC1 antibodies were upregulated in both cancer (45% of patients, n = 58) and IBD cases (28% of patients, n = 39). The authors conclude that this observation suggests the core 3-MUC1 glycoform may be exposed and immunogenic in gastrointestinal chronic inflammatory lesions.

More recently, the link between IBD and the gut microbiome has been explored. It is well-established that the composition of the gut microbiome changes with disease state, among other factors. In their 2020 study, Kappler *et al.* probed changes to both the gut microbiome and to the glycan-binding antibody repertoire in patients with CD (n = 23) or UC (n = 17) and healthy controls (n = 20).[314] To investigate antibody reactivity in each group, arrays containing 220 human milk oligosaccharides were constructed. This class of glycan shares many epitopes and structural similarities to mucosal glycans, making milk oligosaccharides a suitable proxy. Serum screening from each of the groups revealed higher levels of IgG against fucosylated oligosaccharides in CD patients compared to controls. This trend was not observed in UC patients. These fucosylation-targeting IgG were also tested against a consortium of intestinal microbiota, and *Bacteroides stercoris* was found to be significantly increased in IBD patients (interestingly, in both CD and UC patients). The authors also showed that *B. stercoris* is a fucose-displaying microbe. Taken collectively, these findings indicate that the heightened levels of fucose-targeting antibodies in CD patients may be due to the increased presence of fucose-displaying bacteria in the CD gut microbiome.

6.7. Xenotransplants, implants, and ABO-incompatible organ transplants

Glycans play a major role in xenotransplantation, with anti-glycan antibodies mediating reduced xenograft survival and stability in humans.[315–318] Nanno *et al.* investigated anti-glycan IgM and IgG responses in naïve cynomolgus macaques,[319] as well as before and after adult porcine islet xenotransplantation.[320] Naïve monkeys demonstrated highest IgM and IgG binding to α-Gal when presented on a Lac core structure (Galα1-3Galβ1-4Glc, iGb3) and lowest binding when presented on LacNAc (Galα1-3Galβ1-4GlcNAc). Binding was also observed against Tn, TF, GM2 glycolipid, and Sd^a antigen.[319] After transplantation, IgM was elicited against α-Gal, Sd^a, GM2 antigens, or LeX antigen, as well as against Galβ1-4GlcNAcβ1-3Galβ1 and *N*-linked glycans with Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ.[320] For comparison, the repertoire of anti-α-Gal antibodies has also been investigated by glycan microarrays in wild-type[321,322] and in α-Gal-KO mice.[323]

Blixt *et al.* investigated anti-glycan antibody responses in T1D patients transplanted with fetal pig islets. It was found that anti-glycan antibodies increased after transplantation, in particular against the Forssman antigen. Responses were also observed to α-Gal. Glycans with β3-linked Gal similarly elicited a response, as did non-β1-3-linked and non-oligomannosyl β-GlcNAc compounds. There was inter-individual variability in the kinetics of the mounted antibody response, and some patients developed reactivity against Galα1-3LeX and several structures terminated with Neu5Gc after transplantation.[324]

Using a 406-component CFG glycan microarray, Bello-Gil *et al.* screened serum from baboons before and after receiving a red blood cell injection, heart xenotransplantation, or kidney xenotransplantation from transgenic pigs.[325] The authors identified numerous antibodies—each with a different glycan target—present at high levels before transplantation. Notably, many of these antibodies further increased in reactivity after transplant, with antibodies targeting the hyaluronan disaccharide showing the greatest increase relative to pre-transplant levels. To assess the specificity of hyaluronan-targeting antibodies, the authors developed a hyaluronan microarray including hyaluronan oligosaccharides of various lengths, from 2 to 40 saccharides. Antibody reaction strengthened with increasing length of hyaluronan oligosaccharides, especially those between 28 and 40 saccharides long, which the authors conclude is the primary target of hyaluronan-binding antibodies. This work points to a potential role for these antibodies in xenograft rejection and to their potential as a therapeutic target.

Skin harvested from commercial pigs is widely used as a dressing to support healing of skin burns. Pig grafts facilitate rapid healing and are normally viable for up to five days after application. Scobie *et al.* used glycan microarrays to investigate human anti-glycan IgG in serum samples collected from burn patients up to 34 years after they were transiently treated with pig skin, in comparison with age-matched control burn patients that were not treated with pig skin. The analysis revealed sustained antibody responses long after the treatment, with persistent and high IgG reactivity against Neu5Gc glycans. The sialoglycan microarray revealed little to no reactivity to Neu5Ac-glycans, but strong binding to multiple Neu5Gc-glycans, with preference for Neu5Gcα2-6LacNAc/Lac, and Neu5Gcα2-3 linked to core 1 *O*-glycan. High and sustained

anti-Neu5Gc IgG and IgM responses in burn patients, but not in controls, were also confirmed by ELISA against a pool of Neu5Gc-glycoproteins. This analysis had demonstrated the stability of anti-Neu5Gc antibodies over many years in these patients and suggests that they represent a barrier for long-term tolerance of pig xenografts in humans.[326]

In Sections 5.3.3 and 6.5, we have discussed the application of ATG to treating cancer and type 1 diabetes, but ATG is commonly used in allograft recipients as well. ATG's expression of the xenoantigens α-Gal and Neu5Gc[327] makes it a valuable induction treatment for allograft recipients. As seen in cancer and T1D patients treated with ATG, graft recipients experience a shift in their glycan-binding antibody repertoires. Such changes in repertoires of ATG-induced anti-Neu5Gc IgG and IgM have been observed in kidney allograft recipients[328] and recipients of a kidney graft after nonrenal organ transplantation.[329]

Neu5Gc is also expressed on biodevices implanted in human patients. Animal-derived bioprosthetic heart valves are made of tissues from pigs, cows, or horses. It had been shown that these commercial implants express high levels of Neu5Gc,[133] even more than 10 years after implantation.[316] A large longitudinal multicenter study tracked ∼5000 serum samples of patients before and up to 15 years after receiving bioprosthetic heart valves implants. It was shown that anti-α-Gal and anti-Neu5Gc IgG had both increased by one month after implantation. While anti-α-Gal IgG dropped after 6 months, anti-Neu5Gc IgG remained high even two years after implantation. This was validated by glycan microarray analysis of selected recipients *versus* control patients, also showing an overall increase in Neu5Gc-glycans recognition, as observed for ATG-induced antibodies.[328,329]

Anti-glycan antibodies also play a key role in ABO incompatible organ transplants. A 2016 study used a glycan array composed of blood group A, B, and H subtypes (ABH epitopes displayed on different carrier glycan chains) to assess the fine specificity of anti-blood group antibodies in serum.[98] Antibodies to blood group A and B subtype II were absent in patients who were tolerant to an incompatible transplant, demonstrating that antibodies to different subtypes have distinct roles in transplants. A more recent follow-up study concluded that antibodies to blood group A subtype II were most impacted by post-transplant antibody depletion therapies such as therapeutic plasma exchange or ABO-A-trisaccharide immunadsorption.[330]

6.8. Nickel exposure

Although nickel is a widespread component of many everyday objects, occupational exposure to extremely high levels of nickel is linked to lung cancer, nasal cancer, and contact dermatitis. Vuskovic *et al.* used a 380-component glycan microarray to profile plasma samples from individuals who were exposed to nickel at various levels ($n = 89$).[301] Analysis of plasma IgA/G/M revealed binding to lactose-containing (Galβ1-4Glc) glycans modified with either glycine or two amino acids as a spacer. This suggested that there is a potential anti-glycan signature associated with nickel exposure.

7. Conclusions and perspectives

Glycan microarrays enable rapid and comprehensive profiling of serum anti-glycan antibody populations. This technology has been used extensively over the last 20 years to study repertoires of healthy subjects, define disease-specific anti-glycan antibody subpopulations, and identify changes that occur due to vaccination, treatment, or disease. While glycan microarrays have had a major impact, there are numerous opportunities to improve the technology, enhance our understanding of endogenous anti-glycan antibodies, and identify useful biomarkers.

One of the most pressing needs in the field is the expansion of glycan arrays: current designs contain only a small fraction of the diversity found in nature. Therefore, only a subset of endogenous anti-glycan antibodies is measurable to date, and many important antibodies may go undetected due to the absence of a suitable target glycan on an array. For example, current glycan microarrays have very few microbial glycans relative to the plethora of different structures found in nature. Because these microbial glycans are foreign to the immune system, the body likely produces antibodies to many of them, but our ability to measure them is limited. In addition to structure, the presentation of the glycans on the array surface significantly influences recognition. A better understanding of these effects will aid in the optimization of presentation strategies, enabling better profiling of serum antibody populations. The continued expansion of glycan array diversity is critical for improving the technology and more fully evaluating antibody repertoires.

Multiplexing strategies are also valuable in achieving comprehensive profiling. For example, the ability to simultaneously measure different isotypes, subclasses, and glycosylation states of serum antibodies substantially increases the information gained from microarray profiling experiments. Further integration with other data—such as repertoire sequencing data, cytokine profiling, and immune cell profiling—would provide a systems-level understanding of serum anti-glycan antibodies and how they relate to immune function.

While our understanding of endogenous anti-glycan antibodies has significantly improved in recent years, there are still major gaps in our knowledge. First and foremost, we know very little about the relationships between specific anti-glycan antibodies and immune function. While microarrays can establish that endogenous anti-glycan antibodies bind to a synthetic glycan antigen, the natural glycan target *in vivo* often remains unknown. Even when these antibodies can be associated with a particular microbe or target, there is much still unknown regarding their roles in immunity and human health. Determining whether such antibodies are helpful, harmful, or irrelevant to the host remains difficult.

Many of the fundamental properties of endogenous glycan-binding antibodies are poorly characterized. For example, little

is known about the affinities and specificities of most individual endogenous antibodies. Are most antibodies highly selective for a particular carbohydrate epitope, or are they polyreactive? Are the antibodies that bind to a particular component on a glycan microarray oligoclonal, or are there many different antibodies being captured? Relationships between sequences and binding properties are also not well understood.

This lack of basic information limits our ability to identify anti-glycan antibody subpopulations of interest, design arrays tailored to specific antibodies, or predict carbohydrate binding properties from sequencing data. Additionally, we are only beginning to understand how genetic, environmental, and other factors influence anti-glycan antibody repertoires. Better characterization of normal repertoires and the factors that affect them would substantially improve our ability to identify abnormal anti-glycan antibody subpopulations.

One of the main incentives for profiling serum anti-glycan antibodies is the potential to identify useful biomarkers for early detection, diagnosis, and prognosis of disease. Notwithstanding the work done in pursuit of this aim that is summarized in this review, many diseases and conditions have not been investigated using glycan microarrays. For example, anti-glycan antibodies have yet to be profiled for most microbial infections, autoimmune disease, vaccines, and orphan diseases. Moreover, little is known about the effects of various types of immune suppression on anti-glycan antibody levels. Considering these factors, it is clear that the full potential of glycan microarray technologies for biomarker discovery has yet to be realized.

Conflicts of interest

There are no conflicts to declare.

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