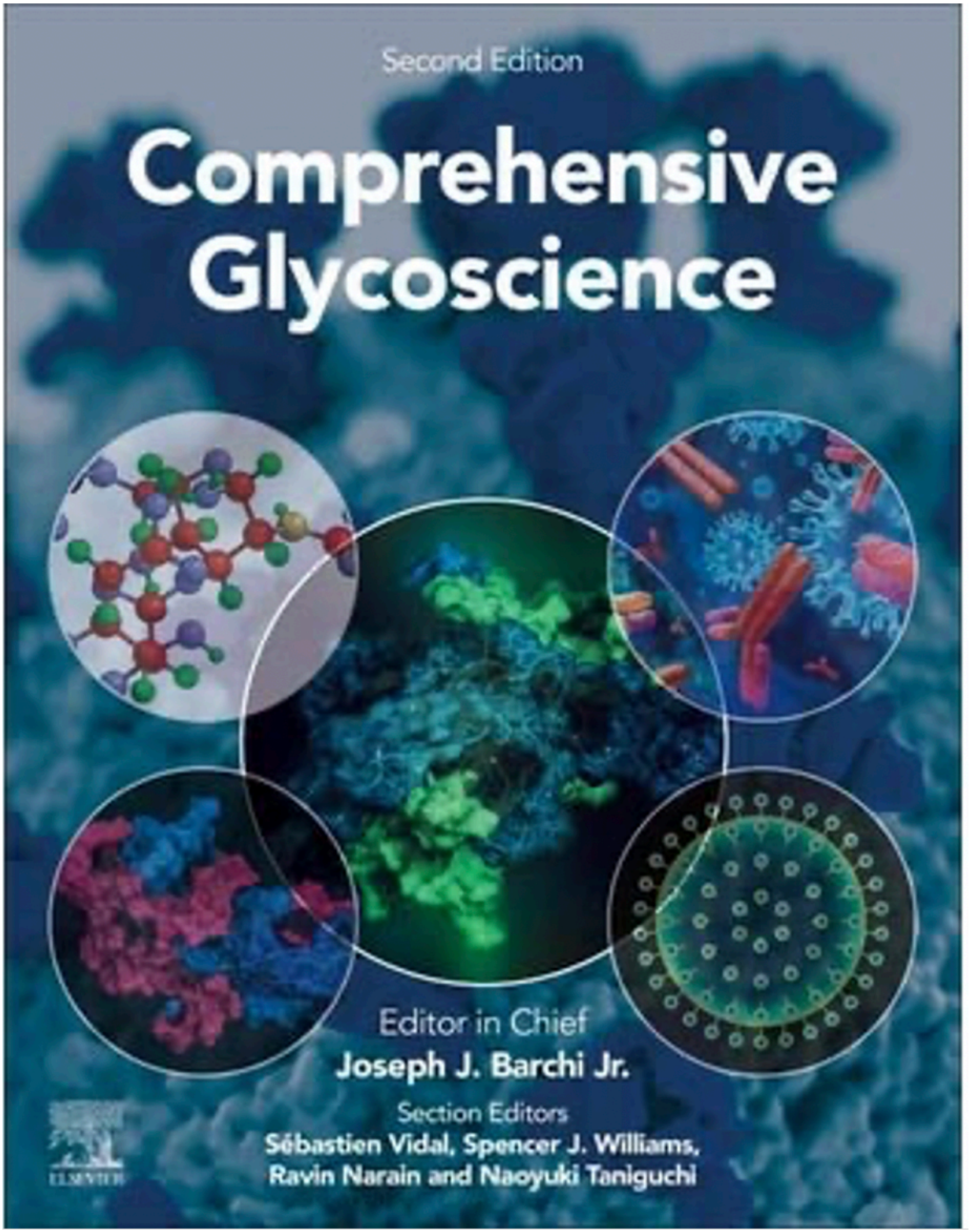
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Systems Glycobiology: Immunoglobulin G Glycans as Biomarkers and Functional Effectors in Aging and Diseases

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Glossary

Biological age Measure of health and functionality of a living organism, determined by the combination of genetic background, environmental factors, and the lapse of time.

Inflammaging Chronic low-grade sterile inflammation characteristic for the elderly, induced by long-term stimulation of the immune system by external (pathogens, commensal organisms, etc.) and internal (DNA damage, cell metabolism byproducts, etc.) stimuli.

Intravenous immunoglobulin (IVIg) Therapeutic preparation consisting of pooled immunoglobulin G from thousands of healthy donors; used to treat antibody deficiencies, some autoimmune diseases, and other immune disorders.

Abbreviations

ACPA	Anti-citrullinated protein antibody
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
Asn	Asparagine
B4GALT1	β-1,4-Galactosyltransferase 1
BCR	B cell receptor
CDC	Complement-dependent cytotoxicity
CE	Capillary electrophoresis
CGE-LIF	Capillary gel electrophoresis with laser-induced fluorescence
CpG	5′ Cytosine-phosphate-guanosine 3′
CVD	Cardiovascular disease
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcRn	Neonatal Fc receptor
FcγR	Fcγ receptor
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
GlcNAc	*N*-acetylglucosamine
HDFN	Hemolytic disease of the fetus and newborn
HFD	High-fat diet
IgG	Immunoglobulin G
IVIg	Intravenous immunoglobulin
J-chain	Joining chain
LC	Liquid chromatography
mAb	Monoclonal antibody
MS	Mass spectrometry
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
TNF	Tumor necrosis factor
UHPLC	Ultra-high-performance liquid chromatography

1 Introduction

Immunoglobulin G (IgG) is one of the key molecules of the human immune system, having evolved in vertebrates as a part of adaptive immunity[1] – our specialized and complex defense system against pathogens and parasites. It is involved in the key features of adaptive immunity: discrimination of self versus non-self, the specific response against many of a broad spectrum of substances (antigens), and specific immunologic memory. The function of IgG is reflected in the structure of this highly versatile protein: the variable fragment antigen-binding (Fab) part aims for specific and high-affinity antigen binding, while the fragment crystallizable (Fc) part interacts with various effector molecules, resulting in immune activation and target destruction by several distinct immune pathways. This soluble effector thus provides an essential piece of the immunological puzzle – it serves as a link between the adaptive and innate immune responses.[2]

However useful in our response against pathogens, whether it occurs through natural infection or immunization through vaccination, this potent molecule can also become dangerous if targeted against our own antigens, giving rise to a plethora of

autoimmune diseases, in which autoantibodies, in addition to auto-reactive T cells and a whole range of other immune system components, can be more or less involved in the disease pathogenesis and progression.[3]

Being placed at such an important crossroads on our multidimensional immune map, while signal relaying, IgG also plays a role of the regulator of the upstream (through feedback) and downstream immune processes, most notably inflammation.[4]

IgG's unique properties have been vastly exploited for therapeutic purposes in the form of monoclonal antibodies (mAbs) and intravenous immunoglobulin preparations. Since the first mAb targeted against CD3 was approved for transplant rejection by the United States Food and Drug Administration in 1986, mAbs have risen to the globally dominant class of biologicals to date, with the market expected to continue growing in the next decade.[5] They are prescribed for different indications, ranging from oncological and chronic inflammatory to immunological, cardiovascular, and infectious diseases,[6] with the extent of on- and off-label indications ever increasing.

With this pivotal role in our immune defense, disease pathology, and therapy, in combination with its omnipresence in research applications, it is obvious why the biomedical and pharma community alike continue to invest a lot of resources to advance the understanding of IgG functionality. Antigen specificity, diverse effector functions of IgG subclasses, and prolonged half-life of IgG3 illustrate the translation of minute structural differences to large-magnitude downstream effects on the IgG function, an effect easily applicable to the structural variation of IgG glycans.

Attachment of glycans to the Fc portion of the molecule[7] and their link to the modulation of its biological functions was reported as early as 1970s.[8–11] However, due to their structural complexity and the lack of sophisticated analytical techniques, IgG glycans have only entered the scientific spotlight relatively recently, the most research-intensive period starting about 15 years ago.[12,13] Up to now, close to 3000 publications have been published related to IgG glycans, with about half of them in the last 10 years. This chapter aims to show it is no accident that IgG is the most studied glycoprotein to date, a trend that seems to be picking up.[13] This chapter will focus on the role of IgG glycans in the molecule's function and their biomarker potential in a personalized approach to medicine and healthy aging.

As is the case with most glycoproteins, glycans are enzymatically added to the nascent IgG molecules through a sequential co- and post-translational process starting in the endoplasmatic reticulum and finishing in the Golgi apparatus of the antibody-secreting plasma cells.[14] Currently, there is an ongoing debate whether and under which immunological circumstances IgG glycans can be modified post-secretion extracellularly in the bloodstream by serum glycosyltranserases.[15,16]

Glycans comprise about 15% of IgG's weight,[17] and their importance is easily discerned from the fact that IgG deglycosylation results in a partial or complete loss of function.[17–19] They represent a structurally and functionally integral part of the IgG molecule, significantly affecting its stability, serum half-life, and effector functions.[20,21] As will be explained in more detail later, our serum contains probably more than a thousand IgG glycosylation variants at any given time, partly arising from alternative glycosylation – a phenomenon reflecting a wide array of different glycan structural variants that can decorate the same glycosylation site. All of the above triggered immense interest in IgG glycosylation, which directed researchers from the initial lectin analyses towards the development of robust and sophisticated analytical methods. The first results coming from studies performed by methods based on the principles of liquid chromatography, mass spectrometry, and capillary electrophoresis indeed confirmed the immense potential for scientific discovery and medical benefit in this research area, which in turn further spurred the method development. Today the high-throughput methods based on all three of the mentioned principles enable for quick acquisition of a large amount of data on IgG glycan composition. While high-throughput analyses have their own challenges and there is always room for improvement, the focus of the field is now shifting towards the development of bioinformatics methods for the integration of the acquired data into large existing frameworks containing other -omics data and thus enabling IgG glycomics to enter the field of systems biology and precision medicine as a clinical biomarker.

The seminal papers on IgG glycosylation by Parekh et al. showed the associations of IgG glycopattern skewing towards a higher content of agalactosylated IgG with rheumatoid arthritis (RA), primary osteoarthritis, and aging.[22,23] This was followed by an increasing number of epidemiological studies with ever-higher numbers of subjects, finally exploding in the last 10 years in parallel with the development of high-throughput methods for IgG glycome analysis, resulting in studies sometimes including thousands of participants.[24–30] We have learned that IgG glycome composition associates with aging, hormonal and diseased status, and much more.[31] Patterns seemed to emerge. It appears now that many diseases with underlying inflammatory processes share a similar IgG glycosylation pattern (mostly a lower abundance of galactosylated and sialylated structures, sometimes accompanied by a higher abundance of structures containing *N*-acetylglucosamine) often also associated with disease severity, and reverted upon successful therapeutic treatment. Notably, this pattern is also associated with increasing age.[32] It has become evident that IgG glycome composition is under significant influence of genes,[33,34] but also environmental factors and lifestyle.[24,35–37] Researchers conducting intervention studies on humans and mice interfering with energy intake, weight, dietary supplements, exercise regime, and sex hormone levels have witnessed a change in IgG glycopattern,[38–43] and in some instances, the uncoupling of disease risk factors and disease development – namely, obesity and hypertension.[38]

While we now collectively have information on more than 100.000 individual IgG glycomes, accompanied by more or less detailed metadata, the answer to the main question on IgG glycans still eludes our understanding: What is the reason behind IgG glycopattern changes in many of the described conditions? In addition, many more questions have surfaced: Do skewed glycopatterns or particular IgG glycoforms have functional roles in disease pathogenesis, progression, severity, or therapy response? Or are they merely by-products, reflections of the immunological and metabolic background on the cellular machinery? What is the role of and how does it come by to alternative glycosylation of antigen-specific compared to total IgG? How is IgG glycosylation regulated? How stable is it? What are the details of the interplay between genes and the environment reflected in our IgG glycomes?

(How) can we exploit the knowledge on IgG glycans to optimize the application of biotherapeutics and vaccines, or patient stratification? Can we use it as a biomarker for disease predisposition or development or an irregular rate of any of the physiological processes happening in our body? Can we, by changing IgG glycosylation, affect all of these processes, e.g. slow-down aging, decrease the risk for a disease or alleviate disease symptoms?

At the moment, there are no definite answers. This chapter is written in an attempt to offer an insight into the current pool of knowledge on this topic, suggest the most likely hypotheses, recommend research priorities, and propose study design for the highest impact on this fascinating but extremely complex topic.

2 IgM, IgD, IgA, and IgE glycosylation

Nearly all key molecules in the innate and adaptive immunity are glycoproteins, with the glycan parts playing a role in their stability and protection from proteases as well as mediating their interactions with other molecules.[44] Human serum contains a large proportion of glycoproteins, including secretory products of the adaptive immune system – immunoglobulins.[45] In humans, five classes of immunoglobulins exist: IgG, IgA, IgM, IgD, and IgE. All of the immunoglobulins consist of four domains called chains, two heavy and two light. Their structure can be further divided by function into the Fc fragment, which is important for the antibody effector functions, and the antigen-binding Fab fragment. Naïve mature B-cells that already went through the process of V(*D*)J recombination that determines their antigen specificity express IgM and IgD in the form of membrane-anchored B cell receptors (BCR).[46]

All human immunoglobulin classes are glycosylated, with glycans attached to the conserved glycosylation sites on their heavy chains accounting for between about 2 and 14% of the total molecular weight, depending on the extent of glycosylation.[17] The light chains contain no conserved glycosylation sites, but new glycosylation sites can be acquired in their variable regions during somatic hypermutation.[47] The steric positioning of glycans within the immunoglobulin tertiary structure is thought to strongly influence their processing and final composition, because of the effect it has on the accessibility of the enzymatic machinery involved in their biosynthesis.[17] This is evident in the IgA glycosylation, where triantennary glycan structures are exclusively present on the Fab region,[48] presumably due to steric hindrance of further processing the Fc glycans.[17]

The glycans attached to immunoglobulins are large (about 2 kDa), flexible if not sterically constrained and play crucial structural and functional roles in the immunoglobulins they are attached to - they maintain the molecules' solubility and conformation and participate in and modulate the binding events with various receptors and other ligands, which results in their involvement in a wide array of biological processes.[17]

2.1 Immunoglobulin M

2.1.1 IgM function

With a concentration of 0.4–3.5 mg/mL, IgM is the second or third most abundant immunoglobulin in the human serum (after IgG and sometimes IgA), and the immunoglobulin dominating the early stages of the humoral immune response.[49] Natural IgM is present in the serum even before antigen exposure, and its main characteristic is polyreactivity, which enables it to bind nucleic acids, phospholipids, and carbohydrates.[50] In contrast, immune IgM is secreted following pathogen exposure and is characterized by a much higher antigen specificity.[50] For the IgM produced early in the immune response – before somatic hypermutation and affinity maturation - the affinity for antigen binding is often low for individual binding sites. This is in part compensated by a large number of possible antigen-antibody binding sites (10 for pentamer and 12 for hexamer IgM), a strategy particularly suitable for the recognition of repetitive epitopes, such as polysaccharides in the bacterial cell wall.[49] The polymeric structure and polyreactivity are key properties that enable IgM's different functions, such as protection against numerous viral, bacterial, fungal, and parasitic pathogens by neutralization, opsonization, and complement activation by the classical pathway as well as targeting apoptotic and altered cells for clearance by phagocytes.[50,51]

2.1.2 IgM structure

Secreted human IgM is mostly a multimeric unit existing as a pentamer of equal monomeric units connected to each other by a joining (J-)chain and disulfide bonds, or occasionally a hexamer lacking the J-chain.[52,53] The multimeric structure of the secreted IgM makes it the largest antibody in the human serum,[54] while monomeric units make up the BCR present at the surface of B lymphocytes.[55]

2.1.3 IgM glycosylation

IgM contains five N-glycosylation sites on the heavy chain: located at Asn-171 in the CH1, Asn-332 in the CH2, Asn-395 and Asn-402 in the CH3 and Asn-563 in the CH4; and one N-glycosylation site on the joining (J-)chain.[17,56] Asn-171, Asn-332 and Asn-395 mostly bear complex, prevalently diantennary, core-fucosylated and sialylated N-glycans.[56,57] Asn-402 carries mostly and Asn-563 (only 17% occupied) exclusively oligomannose structures.[17,57] The N-glycosylation site on the J-chain mainly contains sialylated diantennary glycans.[56] Although IgM glycans seem to be functionally important, the details of IgM glycosylation are not nearly as well explored as those of the IgG glycosylation. They do, however, appear to be necessary for proper IgM secretion.[58] Due to the pentameric structure most of IgM glycans are thought to be inaccessible upon antigen binding.[56] However, in the absence of

antigen binding by the IgM pentamer Fab regions, the complex glycans might allow IgM to agglutinate microorganism in the serum.[56] Recently, sialylation has been shown important for the IgM-mediated inhibition of T cell responses.[59]

2.2 Immunoglobulin D

IgD is also expressed on the surface of naïve B-lymphocytes.[55] It is secreted into the serum as a monomer and present in a wide range of concentrations, from undetectable to 0.4 mg/mL.[60]

2.2.1 IgD glycosylation

IgD contains three N-linked glycosylation sites: Asn-354 in the CH2 domain, occupied by oligomannose glycans and Asn-445 and Asn-496 in the CH3 domain occupied by complex galactosylated and 2,6-sialylated glycans.[61,62] The N-glycans bound to Asn-354 play a vital role in IgD assembly, structure, and secretion.[63] The extended IgD hinge region contains multiple O-glycosylation sites, but the occupancy of all of them (4 threonine residues and 1 serine residue) is not confirmed.[61,62,64,65] These O-glycans do not seem to play a structural role, however, since complete inhibition of O-glycosylation had no effect of the molecule's assembly and secretion.[63]

For the production of the three remaining immunoglobulin classes (IgG, IgA, and IgE): Upon activation by specific antigens B-cells need to undergo an additional process of isotype switching - class switch recombination.

2.3 Immunoglobulin A

IgA is the main antibody in most external secretions and on mucosal surfaces.[66–69] Its daily production surpasses the production of all other immunoglobulins combined.[66,67] A fraction of it can also be found in the serum, where its concentration ranges from 0.6 to 3.8 mg/mL.[49] There are two subclasses of IgA: IgA1 and IgA2, both of which are glycosylated.[17] An obvious difference in the structure of the two subclasses is a much longer hinge region in IgA1, increasing the molecule's flexibility and sensitivity to bacterial proteases.[66,70] This might explain why the vast majority of serum IgA is of the IgA1 subclass, while the IgA2 predominates in mucosal secretions.[66,70,71]

2.3.1 IgA structure

Both IgA1 and IgA2 can be found in monomeric or dimeric (sometimes even trimeric or tetrameric) form.[66,70,71] Most IgA in the blood is monomeric, while the secretory IgA (sIgA) present in secretory fluids, such as mucosa and milk, is mainly dimeric – with the two IgA molecules connected by the J-chain and the secretory component.[66,70,71] The secretory component is responsible for the secretion of IgA onto mucosal barriers, tears, saliva, milk, sweat, etc.[72,73] It also has anti-microbial properties and offers secretory IgA protection against gastric proteases.[74]

2.3.2 IgA function

A poor opsonin and complement activator by the classical pathway, the main role of IgA is the protection of mucosal surfaces from toxins and pathogens by neutralization or prevention of binding, known as immune exclusion.[70,75] Secretory IgA thus serves as the first line of our immune defense against antigens at mucosal surfaces, the most notable probably being those of the intestinal and respiratory tract.[49] Lately, the roles of serum IgA are also becoming known: by binding to Fcα receptor I expressed on the surface of myeloid cells,[76] it induces pro-inflammatory responses, such as phagocytosis and the release of inflammatory cytokines.[77]

2.3.3 IgA Fc glycosylation

Both IgA1 and IgA2 subclasses contain two highly occupied conserved N-glycosylation sites on their heavy chains: located at Asn-263 in the CH2 and Asn-459 in the CH3 region.[17,78,79] In addition to these, IgA2 contains two or three additional conserved N-glycosylation sites, located at Asn-166 and Asn-211 in the CH1 and at Asn-337 in the CH2 domain.[17,79,80] All these N-glycosylation sites are mainly occupied by complex, mostly diantennary (sometimes triantennary) core-fucosylated and sialylated glycans with some variation in their terminal glycan structures, with inconsistent reports on the differences in the N-glycosylation pattern between the two subclasses,[17,48,80–87] and significant differences in glycosylation between IgA originating from different body fluids.[82]

2.3.4 IgA hinge glycosylation

In addition to the N-glycosylation sites, IgA1 also has up to five occupied O-glycosylation sites in its hinge region.[85,88] Just as glycans linked to the N-glycosylation sites, the glycans linked to the Ser or Thr residues in the O-glycosylation sites are rather heterogeneous –composed of *N*-acetylgalactosamine, galactose, *N*-acetylneuraminic acid (sialic acid), and, in some cases, *N*-acetylglucosamine (GlcNAc).[48,81,83,86,87,89]

2.3.5 IgA Fab glycosylation

About 30% of the serum IgA contains N-glycans in the Fab region, mainly complex diantennary and triantennary sialylated structures.[48] Indeed, almost all of the molecule's triantennary structures originate from its Fab region,[48] presumably due to easier

access for the branching enzyme *N*-acetylglucosamine transferase IV.[17] Additionally, some O-glycosylated structures are reported for the Fab region.[48]

2.3.6 IgA joining chain and secretory component glycosylation

Secretory IgA contains considerably more glycans than serum IgA because both the J-chain and the secretory component are amply glycosylated.[87,90–93] The J-chain contains a single N-glycosylation site, characterized by an unusually high level of 2,3-linked sialic acid; and the secretory component contains seven N-glycosylation sites, all of them mostly carrying complex sialylated diantennary glycans, specific by the presence of outer arm fucose.[81,82,87]

2.3.7 Functions of IgA glycans

Although their structure-function relationship is not as explored as is the one of IgG glycans, IgA glycans are, similarly to IgG glycans, considered relevant for the molecule's effector functions because of their capacity to modulate the binding affinity for various receptors.[17] The reports about the effect of their composition on the binding to FcαRI, the key receptor for IgA, remain, however, ambiguous.[48,94,95] It seems clear though, that the composition of IgA N-glycans affects its clearance from the serum, due to the modulation of affinity for asialoglycoprotein receptor on hepatocytes.[94] Next, secretory IgA glycans (including the N-glycans of the secretory component) enable the entrapment of IgA in the mucosa and bind microorganisms to prevent their attachment to the epithelium and thus facilitate their clearance by mucociliary flow or peristalsis.[70,74,96–100] In addition to pathogen binding, the N-glycans of the secretory component and the O-glycans of the extended IgA1 hinge region protect their respective molecular regions from proteases.[101,102] Recently, similarly to IgG, sialylation was described as a modulator of the IgA1 inflammatory capacity - desialylated IgA glycoforms showed stronger inflammatory activity *in vitro*, although the entire underlying mechanism of this phenomenon, including the binding partner of the sialylated IgA molecules, remains unknown.[80] How this finding relates to the fact that an increased level of sialylated IgA is reported in breast cancer[103] is not yet clear.

More is known on the role of IgA glycans in IgA nephropathy, where agalactosylated glycans are considered contributors to disease pathogenesis in two ways: firstly (and more directly) - the agalactosylated O-glycans of the IgA1 hinge region induce the production of autoantibodies that form nephritogenic immune complexes; and secondly - by binding to the MBL, the agalactosylated N-glycans can activate the lectin complement pathway and thus initiate inflammation.[104,105]

Interestingly, age-dependent changes in IgA N-glycopattern similar to the ones observed for IgG (discussed later), but also in O-glycopattern, have been reported.[81]

2.4 Immunoglobulin E

With a concentration of up to 0.05 mg/mL in healthy adults, IgE is the least abundant immunoglobulin in human serum and is mostly explored as the key mediator of mast cell and basophil degranulation in allergies.[106]

2.4.1 IgE glycosylation

It is perhaps not broadly known that IgE is heavily glycosylated with as many as seven N-glycosylation sites.[62,107,108] Of those, Asn-383 in the CH3 domain is unoccupied, Asn-394 in the same domain is decorated by a single N-linked oligomannose glycan (most often containing five mannoses); and the remaining five N-glycosylation sites (Asn-140, Asn-168, Asn-218 in the CH1 domain of the Fab region and Asn-265 and Asn-371 in the CH2 and CH3 domains of the Fc region, respectively) are occupied by complex sialylated diantennary N-glycans.[62,108–110] Similarly to IgG, IgE glycosylation is known to exhibit a significant inter-individual variation and has disease-specific patterns.[111,112] And, again similarly to IgG, although they are thought to be involved in the IgE effector functions, the role of IgE's complex glycans is not yet fully elucidated. They are suggested to contribute to the solubility of IgE in water, preventing aggregation by their hydrophilic properties[113] and, when it comes to the complex glycans in the CH1, to modulation of IgE antigen binding.[109] Recently sialic acid was confirmed as a determinant of the IgE pathogenicity and thus an important regulator of allergic disease.[112] Perhaps not surprisingly, the most relevant seems to be the odd one out though: The oligomannose N-glycan occupying the Asn-394 is required for appropriate folding and is indispensable for IgE effector function, namely for the initiation of anaphylaxis through binding to the high-affinity FcεRI on the surface of mast cells.[109,114] Interestingly, this N-glycosylation site is conserved across all mammalian IgE and is orthologous to the conserved N-glycosylation site of IgG, pointing to its potential analogous relevance.[111,115]

3 Immunoglobulin G structure and function

With a concentration range of 6–14 mg/mL, IgG is the most abundant immunoglobulin in the human serum,[49] representing 75% of all antibodies and 10–20% of total plasma protein.[116]

3.1 IgG structure

Immunoglobulin G is a 150 kDa globular protein consisting of four peptide chains: two heavy chains connected by disulfide bonds, and two light chains - giving it a recognizable structure that consists of three domains connected by a flexible hinge region, often

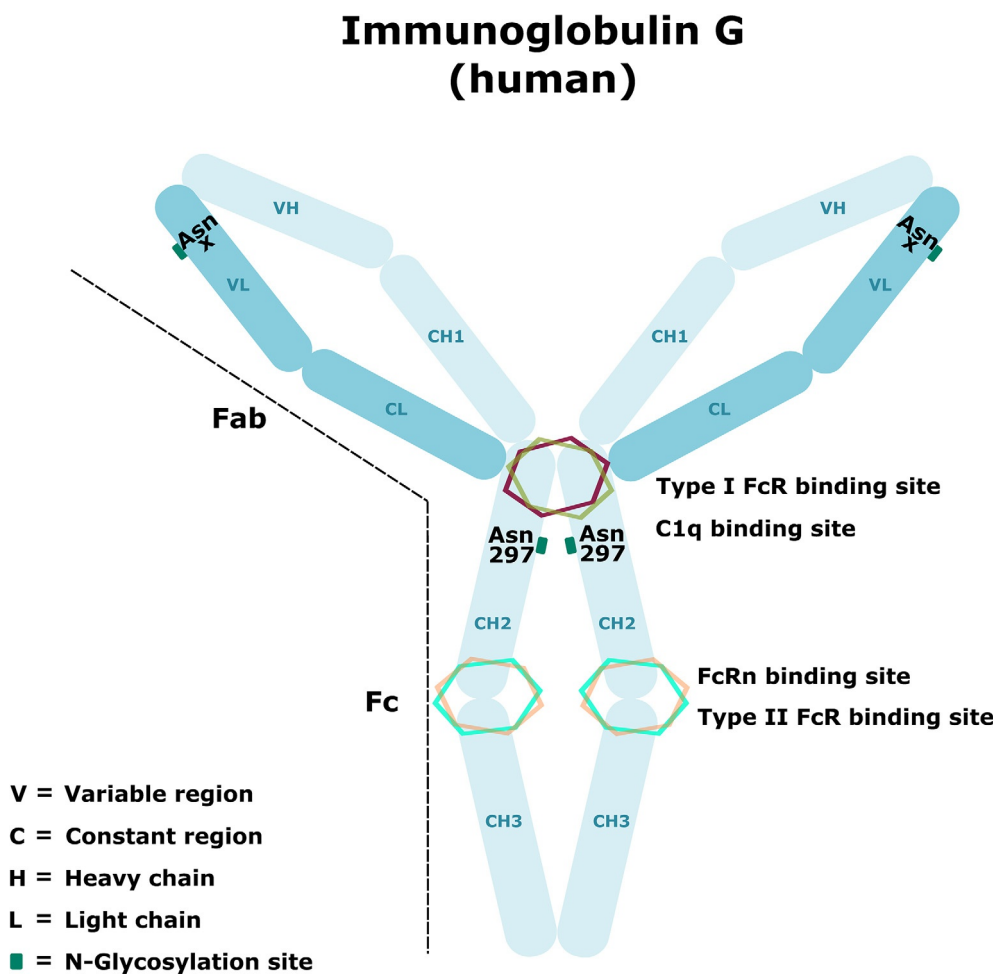


Fig. 1 Human immunoglobulin G N-glycosylation and ligand-binding sites. Each of the two heavy chains contains a conserved N-glycosylation site at the asparagine 297. In contrast, only about 15–20% of IgG molecules contain an N-glycosylation site within one of their light chains. These N-glycosylation sites are not conserved – they appear during IgG somatic hypermutation as a part of fine-tuning the response against the given antigen (affinity maturation). *Asn* = asparagine, *C1q* = complement component 1q, *Fab* = fragment antigen-binding, *Fc* = fragment crystallizable, *FcR* = Fc receptor, *FcRn* = neonatal Fc receptor, *IgG* = immunoglobulin G.

simplified into the characteristic Y-shape (Fig. 1). The structure of IgG is also subdivided into two regions with different functional properties: the Fc region, consisting of the domains of the heavy chains below the hinge, and the Fab region, consisting of the light chains and the domains of the heavy chains above the hinge.[70,117]

3.2 IgG functions

IgG is one of the main effector molecules of the human adaptive immune system, linking it with the evolutionary older innate immunity by the versatile nature of the molecule: the Fab region specifically recognizes and binds an almost unlimited assortment of antigens, while the Fc region binds to various receptors, thus initiating multiple immune effector pathways.[2] The role of the IgG in the immune response is three-fold.

First, through the specific antigen-binding properties of the Fab fragment, it acts as one of the main recognition molecules of the adaptive immunity and can directly neutralize microorganisms and toxins by preventing their binding and/or cell invasion.

Second, through the Fc fragment that interacts with type I and type II Fcγ receptors on the surface on many immune cells (including B lymphocytes, macrophages, neutrophils, natural killer cells, etc.) it can trigger opsonization and phagocytosis of microorganisms (antibody-dependent cellular phagocytosis, ADCP), mediate antibody-dependent cellular cytotoxicity (ADCC) and inhibit or activate other immune cells.[118] Indeed, through the simultaneous triggering of activating and inhibitory Fcγ receptors (FcγRs) co-expressed on the surface of various innate immune cells, such as macrophages, neutrophils, and dendritic cells, IgG effectively sets a threshold for immune cell activation.[119]

And third, through the interaction of the Fc fragment with other components of the immune system (e.g. complement component C1q, the mannose-binding lectin, and mannose receptor) it can activate the complement system and cause lysis of microorganisms and damaged self cells and initiate inflammation (complement-dependent cytotoxicity, CDC).[116,120–122]

3.3 IgG subclasses

IgG exists as four subclasses - IgG1, IgG2, IgG3, and IgG4, which acquired their numbering by decreasing serum concentration.[123] Although their overall structure is similar and the homology at the amino acid level is over 90%,[116] some sequence variation exists in their constant regions, particularly in the hinge region and the N-terminal CH2 domains.[116] Since these are the parts of IgG which interact with various effector molecules, such as type I and type II FcRs, C1q, and the neonatal Fc receptor (FcRn) (Fig. 1), the structural differences in these regions likely affect the binding affinity of various IgG subclasses to their ligands, which in turn translates into differences in their biological properties: half-life, placental transfer, complement activation, immune complex formation, activation of FcγR-expressing cells, etc.[116,124] In addition, antigen type (chemical composition) and immunization route steers the immune response towards preferential subclass secretion: protein antigens usually tend to direct class switching to IgG1 and IgG3 (also IgG4) through T-cell dependent mechanisms, while the polysaccharide antigens, without the help of T-cells, often skew IgG response towards the IgG2 subclass.[116,125]

Interestingly, IgG3 stands out among other subclasses by its extended hinge region (giving the molecule increased flexibility), a short half-life (for most IgG3 allotypes it is only 7 compared to 21 days for other subclasses), the largest number of allotypes, and an emphasized potency in the induction of pro-inflammatory effector functions.[116] IgG4 is unique among the IgG subclasses because of its lack of ability to activate complement and, even more interesting, because of a phenomenon called "Fab-arm exchange" – switching of heavy chains and attached light chains (half-molecules) between two IgG4 molecules, resulting in bispecific but functionally monovalent antibodies.[126,127] This is suggested to be the basis for the anti-inflammatory activity of IgG4 due to the inability to form large immune complexes, a property that can be both desirable and undesirable in a therapeutic setting, depending on the mode of action.[127–129]

3.4 Pathogens fight back against IgG

The importance of IgG in the fight against pathogens is evident in the rich arsenal the bacteria have evolved to fight against this molecule in the form of cell-wall-anchored IgG-binding proteins, such as protein A, G, H, L, M and Sir; secreted IgG-binding proteins, such as Sbi, SibA and Lzp; and IgG proteases, such as *Proteus mirabilis* IgG protease, IdeS and SpeB, which cleave the molecule in the hinge region, thus uncoupling the IgG antigen binging and effector functions.[130] However, it might be less widely known that the list of enzymes that have evolved in bacteria with the aim of IgG glycan hydrolysis is also quite long and points to the importance of IgG glycans in the defense against pathogens: EndoS, EndoE, EndoC/EndoSE, EndoS2, EndoD, SiaC, GpdC.[130]

4 General aspects of immunoglobulin G N-glycosylation

Every IgG molecule contains a conserved N-glycosylation site on the Asn-297 on each of its heavy chains.[131] This site is located in the CH2 domain of the molecule's Fc region, the part of the molecule (CH_2-CH_3) where most of the contacts with the various IgG Fc receptors and ligands take place (Fig. 1).[132] Fc-linked N-glycans are positioned in the cavity between the two opposing CH2 domains of the two heavy chains, thus shielding the hydrophobic surface of the Fc core from the exposure to solvent.[7,133]

Additional N-glycosylation sites can also be introduced in the Fab region of IgG through somatic hypermutation that occurs during affinity maturation in germinal centers: glycans have been observed in the variable region of IgG heavy chain in 15–20% of molecules in healthy individuals (Fig. 1).[47,134]

4.1 Structure of IgG N-glycans

All IgG N-glycans share a common conserved pentasaccharide core sequence consisting of two GlcNAc and three mannose residues branching into two antennae.[135] This core is most often expanded into a heptasaccharide with a GlcNAc attached to each of the mannoses; the heptasaccharide core can be further expanded by the addition of bisecting GlcNac and/or core fucose, and the antennae further extended by the addition of galactose and, following galactose, *N*-acetylneuraminic (sialic) acid.[17] The most complex IgG N-glycan thus consists of 13 monosaccharide units and represents a diantennary core-fucosylated digalactosylated and disialylated structure with a bisecting GlcNAc (Fig. 2). In addition, some oligomannose glycans containing 5 to 9 mannose residues have been observed on IgG N-glycosylation sites.[137] So far, over 30 different N-glycan structures have been observed on the Fab and Fc portions of polyclonal serum IgG (Table 1).[34,137]

When interpreting IgG glycopatterns, for the ease of understanding glycans with similar structural features are sometimes combined into "derived traits." The most often used IgG glycosylation derived traits refer to the cumulative abundance of: agalactosylated (G0), monogalactosylated (G1), digalactosylated (G2), galactosylated (G = G1 + G2), asialylated (S0), monosialylated (S1), disialylated (S2), sialylated (S = S1 + S2), afucosylated (F0), core-fucosylated (F) and bisected (B) N-glycans.

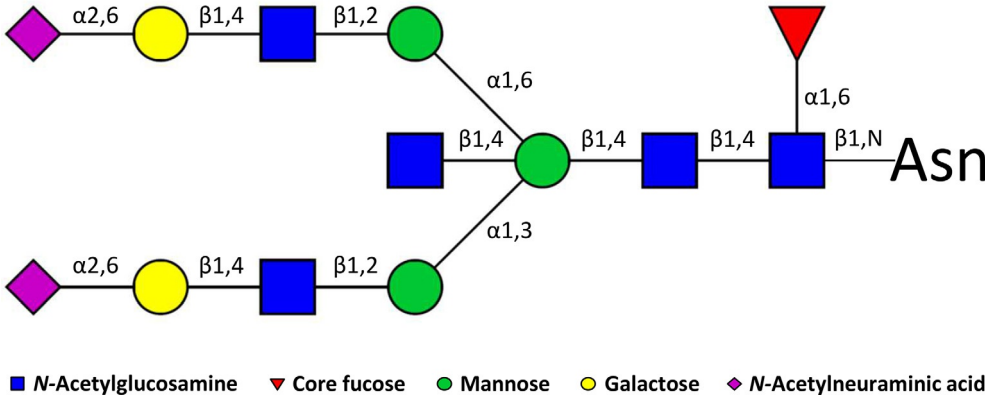


Fig. 2 The most complex immunoglobulin G (IgG) N-glycan. The majority of other IgG N-glycans correspond to this tridecasaccharide with the lack of one or more monosaccharide residues. The notation above the lines connecting monosaccharide units denotes the type of the glycosidic bond. Often the antenna formed in the extension of the mannose α1,6-linked to the central mannose is referred to as the "α1,6 arm," whereas the other antenna is referred to as the "α1,3 arm." The monosaccharide residues are color-coded according to the recommendation by the Consortium for Functional Glycomics.[136]

4.2 Positioning of Fc N-glycans

The core of the Fc glycan is in contact with the inner surface of the CH2 domain, and the α1,6 arm is, likewise, folded alongside the CH2 polypeptide backbone, forming multiple noncovalent interactions with amino acid side chains Phe-241, Phe-243, Lys-246, Glu-258, Asp-265, Tyr/Phe-296 and Asn-297 (Fig. 3).[133,140] The importance of these amino acids is evident from the fact they are conserved across all of the IgG subclasses.[116,141] The other (α1,3) arm extends into the cleft between the two CH2 domains, where it interacts with the α1,3 arm of the N-glycan attached to the Asn-297 of the other heavy chain.[133] The interaction of the N-glycans on the two opposing heavy chains thus keeps the Fc in the "open" conformation needed for type I FcγR binding, which points to their importance in the proper functioning of IgG.[133,140] Indeed, aglycosylated IgG cannot bind type I FcγRs or activate complement through C1q binding, rendering its effector functions either heavily impaired or completely abrogated.[17–19] Reports that a single monosaccharide is sufficient for FcγR binding are still under debate.[142–146]

4.3 Fc glycans affect IgG structure and function

Glycans represent, not only structurally but also functionally, an integral part of the IgG molecule. By modulating binding affinity to IgG's receptors and ligands, glycans affect nearly all biological processes involving IgG: its half-life, clearance and placental transport (through binding to FcRn), modulation of activation of immune effector cells (through binding to type I and type II FcRs) and complement (through binding to C1q and mannose-biding lectin) (Fig. 1).[17,122,133,147–150] In addition to the stated extrinsic effects, IgG glycans also have intrinsic effects on the protein itself, such as maintaining its conformation and solubility.[140,151] The role of Fc N-glycans in the modulation of particular IgG effector functions through the fine-tuning of the Fc conformation has been a subject of detailed exploration and will be discussed in more detail in Section 7.

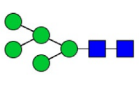
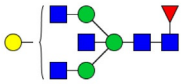
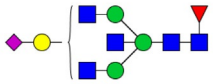
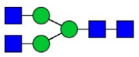
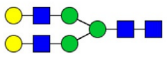
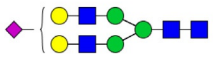
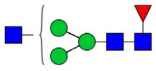
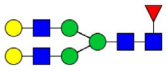
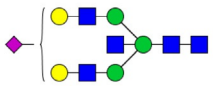
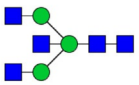
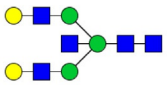
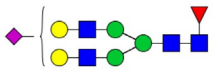
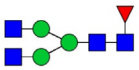
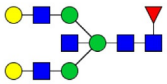
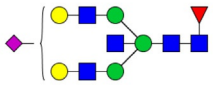
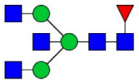
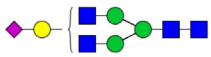
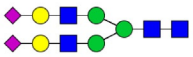
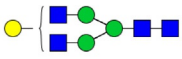
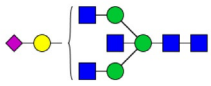
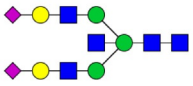
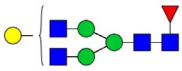
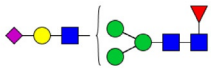
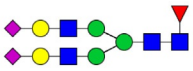
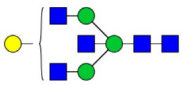
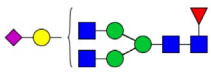
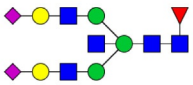
4.4 Asymmetrical Fc glycosylation

Mainly due to the limitations of analytical techniques, the pairing of the N-glycans attached to the two opposing heavy chains has not been extensively studied, although both symmetrically (containing homologous glycans) and asymmetrically glycosylated Fc IgG (containing non-homologous glycans) have been reported in the natural setting.[152] The biological consequences of this phenomenon have not been explored. Of note, the term "asymmetrical glycosylation" is sometimes also used for the IgG molecules with only one of the two conserved N-glycosylation sites occupied. These antibodies are also referred to as "hemiglycosylated." This phenomenon has only been observed in *in vitro* produced antibodies, which retain little of the IgG biological activity.[153,154]

4.5 Fab versus Fc glycan composition

Although the pool of Fc and Fab N-glycans consists of the same structures (Table 1), they differ significantly in their relative abundances. Almost all Fab glycans (94%) are galactosylated at both arms, with about 40% of them bearing mono- and 52% di-sialylated glycans.[134] This is in stark contrast with Fc glycans, where only 1% is di-sialylated.[134] Additionally, the level of bisection is about 3 times higher in the Fab region, with about 45% of Fab glycans carrying a bisecting GlcNAc compared to 10% in the Fc region; and the level of fucosylation significantly lower, with only about 70% glycans carrying a core-fucose, in contrast with over

Table 1 Prominent N-glycan structures present on human immunoglobulin G.

 M5	 FA2BG1	 FA2BG1S1
 A2	 A2G2	 A2G2S1
 FA1	 FA2G2	 A2BG2S1
 A2B	 A2BG2	 FA2G2S1
 FA2	 FA2BG2	 FA2BG2S1
 FA2B	 A2G1S1	 A2G2S2
 A2G1	 A2BG1S1	 A2BG2S2
 FA2G1	 FA1G1S1	 FA2G2S2
 A2BG1	 FA2G1S1	 FA2BG2S2

The actual number of glycans is increased by the existence of structural isomers (3 and 6 arm) denoted by brackets.
Mx = number of mannose residues, F = core fucose, Ax = number of antennae, B = bisecting N-acetylglucosamine, Gx = number of galactose residues, Sx = number of sialic acid residues.

90% of Fc glycans being core-fucosylated.[134] There are also reports on more high-mannose glycans in the Fab region, depending on the position of the N-glycosylation site in the variable region of the heavy chain.[155–157]

The difference in the level of processing seen in Fab and Fc N-glycans, reflected in different relative abundances of glycoforms, is thought to arise from higher accessibility to glycosyltransferases of the Fab glycans, which are highly exposed, in contrast to the Fc glycans that are positioned at the inner face of the two CH2 domains.[134] This concept, already mentioned for the composition of IgA Fab versus Fc glycans, is confirmed by lectin and antibody binding assays.[17,48,158,159] However, it does not explain the lack of tri- or tetraantennary N-glycans on IgG Fab.

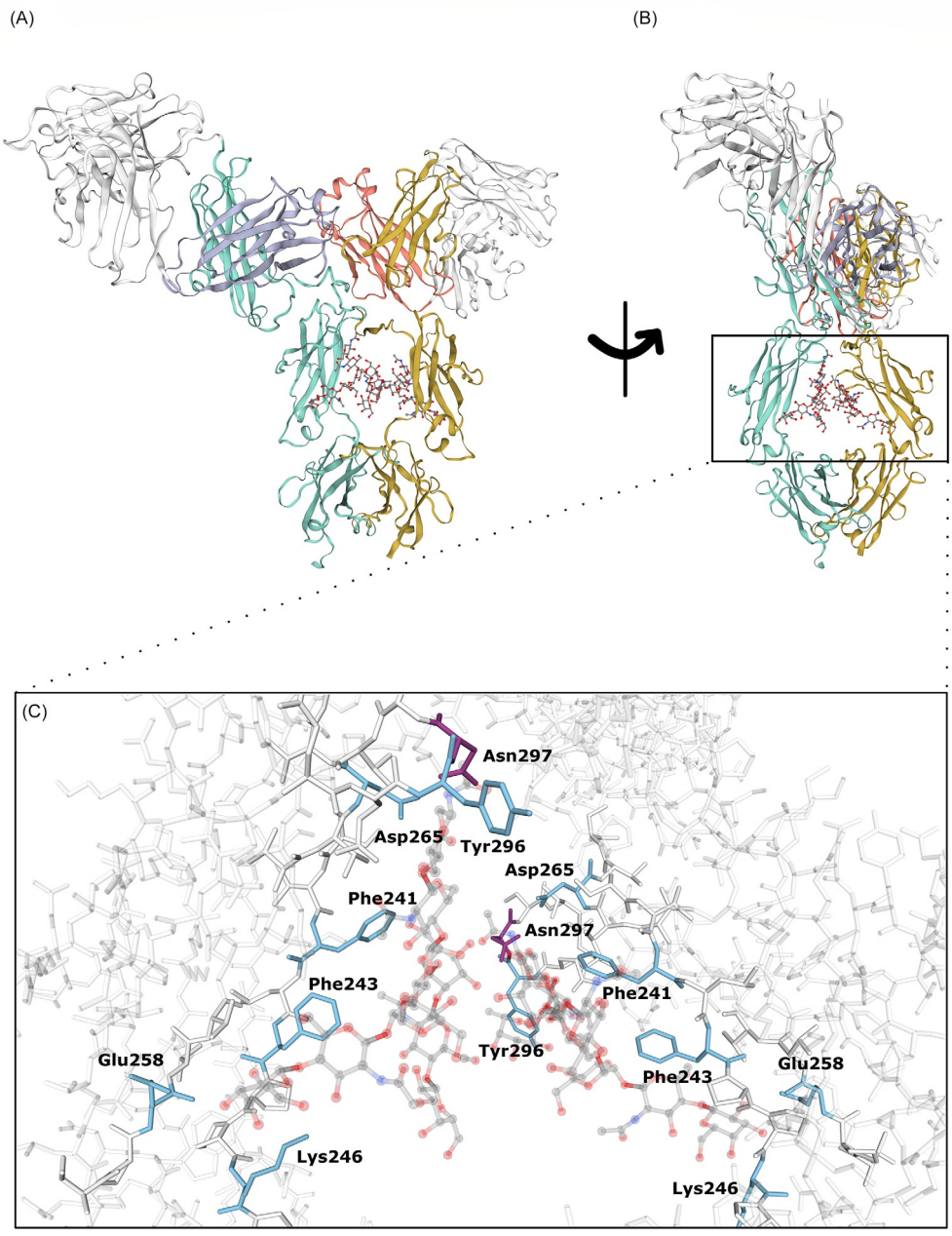


Fig. 3 The immunoglobulin G Fc glycans are positioned in the cavity between the CH2 domains (A and B), forming multiple noncovalent interactions with the polypeptide backbones of the two heavy chains (C). SWISS-MODEL Workspace[138] was used for depicting human IgG1 b12[139] (UniProtKB accession numbers P01857 and P01834 - heavy and light chain, respectively). Turquoise and yellow = constant regions of heavy chains, lilac and orange = constant regions of light chains, grey = variable regions of heavy and light chains. Cartoon model = immunoglobulin G protein backbone, ball-and-stick model = N-glycans.

4.6 Fab glycans affect IgG structure and function

Just as the Fc glycans can introduce subtle changes in the IgG structure, Fab-linked N-glycans can affect IgG's biological properties and functions, such as half-life, solubility, stability, antigen binding, aggregation/immune complex formation, and downstream immune response effector functions.[134,157,160–164]

4.7 Asymmetrical Fab glycosylation

Similar to the Fc region, the Fab region can also be asymmetrically (hemi)glycosylated, i.e., having only one Fab arm glycosylated, but unlike for the Fc glycans, this has also been observed in natural IgG molecules as early as 1980s and confirmed in more recent

studies.[165–168] Such a glycosylation pattern imparts profound consequences on the function of IgG molecules: The steric hindrance imparted by the single glycan in one antigen-binding site dramatically lowers the binding affinity for antigen in this arm, making them behave as functionally "univalent" antibodies. This prevents the formation of large immune complexes and in turn, various effector functions.[169]

The distinct patterns that both the Fab and Fc N-glycans exhibit in various pathophysiological conditions and their potential immunomodulatory roles will be discussed in detail in Section 8.

4.8 IgG3 O-glycans

In addition to the N-glycans, each of the heavy chains of the molecules belonging to the IgG3 subclass contains up to three O-glycosylation sites in its extended hinge region.[170] About 10% of the threonines within the proline-rich triple repeat sequence are occupied with mainly non-, mono- and disialylated core I type (*N*-acetylgalactosamine-galactose) O-glycans.[170] The function of these glycans has not been established yet, but based on the knowledge gathered from the glycans on other IgG subclasses it is expected they might: (1) protect the molecule from proteolytic degradation by bacterial or endogenous proteases, (2) contribute to the maintenance of the extended conformation, or possibly (3) be involved in pathological processes, as is the case with IgA hinge O-glycans. Additionally, based on the fact that asymmetrical O-glycosylation has been reported for the mouse IgG2 hinge O-glycans,[171] the same can be expected for human IgG3, but this remains unconfirmed.

5 IgG glycosylation analytics

Large heterogeneity in glycan structural composition, presence or absence of Fab glycans, and asymmetric glycosylation in combination with four subclasses theoretically result in thousands of different IgG glycoproteins in human serum. Due to the lack of analytical methods that could address this daunting complexity, glycomics has been lagging significantly behind genomics and proteomics. To address these deficiencies, IgG-focused glycoscience, in particular, has exploded in the last 10–15 years.[12]

5.1 Lectins

Initially, an IgG glycome was mostly analyzed using techniques based on lectin binding, such as lectin chromatography, lectin enzyme-linked immunosorbent assay (ELISA), lectin blotting, and lectin immunofluorometric assays (IFMA).[22,172–174] Lectins used in IgG glycan analytics are proteins mostly expressed in plants, which specifically recognize glycans sharing distinct structural features.[175] Although they have been known since the late 19th century as phytoagglutinins (plant hemagglutinins), they became known under their contemporary name and started attracting more attention in the 1970s with the realization they can be extremely useful as glycoanalytical and glycopreparative tools.[176,177] Although now considered obsolete by some, lectins deserve to be credited for their immense contribution in advancing glycobiology. Indeed, they enabled some of the important discoveries even in the "modern" era of IgG glycan analysis, e.g. using the *Sambucus nigra* agglutinin lectin to delineate the importance of sialylated IgG for the anti-inflammatory activity of intravenous immunoglobulin (IVIg) preparations.[178] Some of the lectins most often used for human IgG glycome exploration, with their respective specificities, are: The already mentioned *Sambucus nigra* agglutinin (SNA) – recognizing α2,6-linked terminal *N*-acetylneuraminic acid; *Ricinus communis* agglutinin I (RCA I) – recognizing terminal galactose; *Phaseolus vulgaris* erythroagglutinin E (PHA-E) – recognizing terminal galactose and bisecting GlcNAc; *Griffonia simplicifolia* lectin II (GSL II) – recognizing agalactosylated N-glycans; *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA) and *Aleuria aurantia* lectin (AAL) – recognizing core fucose; and concanavalin A (Con A) – recognizing high-mannose.[173,178–181] Although not appreciated by some due to their complex affinity patterns, which sometimes cause cross-reactivity, lectin-based methods remain a promising tool in IgG glycoanalysis when combined with upgraded modern technological approaches, e.g. lectin microarrays.[179,182]

5.2 Advanced analytical tools

The interest in IgG glycans sparked by the initial studies performed in the 1980s and the beginning of the new era in IgG glycobiology in the 2000s triggered the development of analytical tools capable of elucidating the structural complexity of IgG glycans.[12] In addition, the need for large-scale analyses with greater statistical power called for high-throughput, sensitive, robust, reproducible, affordable, relatively simple, and time-effective glycoanalytical techniques.[183]

Methods that emerged as a result of this scientific endeavor and are used for IgG glycoanalysis today are largely based on capillary electrophoresis (CE), liquid chromatography (LC), and mass spectrometry (MS) and "merely" modified or optimized for glycan or glycopeptide analysis.[184–186] Each of the analytical methods has its advantages and disadvantages, in terms of glycan separation and structure determination as well as equipment price and necessary time and expertise. Currently, the vast majority of studies on large sample sets are performed by ultra-high-performance liquid chromatography (UHPLC) for total IgG glycans and liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) for Fc IgG1 (and, occasionally, other subclasses).[24,26–28,34,187] For studies on low sample numbers and requiring a high level of structural detail, the best approach is to combine several complementary techniques.

5.3 Ultra-high-performance liquid chromatography (UHPLC) and capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF)

Following the enzymatic release and fluorescent labeling with 2-aminobenzamide, 2-aminoacetic acid, or procainamide for UHPLC and 8-aminopyrene-1,3,6-trisulfonate acid for CGE-LIF, particular structures or structural groups of N-glycans (including isomers – enabling for arm-specific information collection) are separated into chromatographic or electrophoretic peaks due to their differential retention time.[188] By measuring the fluorescence intensity, these methods offer the information on the abundance, but (if used standalone) not on the content of particular glycan peaks.[188] This can be sufficient for the analysis of a large number of samples in situations when the content of each glycan peak can be reasonably assumed to remain stable, such as large-scale epidemiological studies on homogenous demographic groups. Indeed, studies involving hundreds and thousands of samples have been performed using these methods, mostly UHPLC.[26,27,34] UHPLC and CGE-LIF are mainly used for glycans enzymatically released from the whole IgG molecules,[26,27,34] but can also be applied for the glycans released from its Fab and Fc portions.[148,189] Although UHPLC IgG glycan analysis is robust, reproducible, relatively simple, and widely used in the high-throughput mode,[190] it is expected that CE-based methods will be the real champion of the high-throughput IgG glycan analysis in the future because they allow multiplexing and simultaneous analysis of up to 96 samples by using multi-capillary arrays.[191] In cases where information about the exact structures within a glycan peak is needed, due to incompatibility of non-volatile electrolyte components with MS detection,[192] enzymatic digestion using exoglycosidases with different specificities can be applied for CGE-LIF, with multiple runs for each sample.[193] In addition to exoglycosidase digestion, UHPLC can alternatively be coupled to MS, allowing for glycan peak structure determination by fragmentation in tandem mode.[194]

5.4 MS-based methods

Measuring the intensity over the mass-to-charge ratio (m/z), the MS-based methods offer better structural insight and are becoming a method of choice where more precise structural analysis of IgG glycans is necessary. LC-ESI-MS is routinely used for the analysis of IgG Fc glycopeptides,[78,195] whereas matrix-assisted laser desorption ionization time of flight (MALDI-TOF)-MS is, in addition to Fc glycopeptides, also used for released IgG glycans.[196–199] For the analysis at the glycopeptide level, IgG is enzymatically cleaved by the trypsin protease, which results in tryptic glycopeptides containing nine amino acids surrounding the conserved Fc N-glycosylation site.[78,195] Prior to the MS analysis, glycopeptides are separated by reverse phase liquid chromatography (RP-LC) by their peptide content.[78,195] Since IgG subclasses differ in two out of nine amino acids within their respective glycopeptides, this method allows for a subclass specific analyses of Fc glycans.[78,195] In this way, within one run, N-glycans belonging to each of the subclasses can be analyzed separately. The exception to this is IgG3, which shares its tryptic glycopeptide amino acid sequence either with IgG2 or IgG4, depending on the allotype.[78,200] A glycopeptide-based approach has also been employed for the analysis of IgG3 hinge O-glycans.[170] Moreover, the amino acid sequence difference between IgG and IgA permitted the simultaneous analysis of the glycopeptides belonging to the two immunoglobulin classes, reported recently.[81] Although not at common in epidemiological studies, IgG glycopeptide analysis can also be performed by capillary electrophoresis electrospray ionization (CE-ESI)-MS.[192,201] Unlike the Fc glycans, the Fab glycans can only be analyzed at the level of released glycans,[189] due to the lack of conserved glycosylation sites in the Fab region.[134]

The MS instrumentation and analytics are widely considered more complex than those used for UHPLC and CE. As a consequence, the MS-based methods for IgG glycome analysis usually require a higher level of expertise, particularly when it comes to structure determination by fragmentation.

5.5 Data interpretation

It is important to note that the abundance of glycans is always expressed as a percentage of particular glycan structures within total IgG (or Fab or Fc) glycans for released glycans or within total glycans attached to a particular subclass for subclass specific Fc glycans. The values obtained for each of the subclasses cannot be simply added up to obtain the glycan abundances on the level of total IgG due to the inherent differences in response factors among the four subclasses. This can (and, indeed, does) complicate the interpretation of the biological significance of the results and prevent the comparison of e.g. absolute values of glycoforms present on different IgG subclasses. It is of utmost importance to always keep in mind the methodology used to obtain the results that are discussed and the limitations thereof.[202] Likewise, data obtained through different analytical methodology should only be compared with great care.

Although it is very tempting to interpret the results of an IgG glycan analysis on the level of single glycoforms, this should be done with caution. Since the abundances of particular glycans or structural groups of glycans are expressed as a proportion of total glycans due to normalization by total chromatogram/mass spectrum/electropherogram area or height, a reduced percentage of any particular structure inevitably results in a raised percentage of other structures and vice versa. Although more demanding and perhaps less intuitive, IgG glycosylation should, therefore, also be described on the level of the entire glycosylation profile rather than individual structures alone.

6 Intricacy of IgG glycosylation regulation

6.1 Extreme complexity of IgG glycome

More than 30 structures are documented among the N-glycans released from polyclonal IgG in the human serum (Table 1).[34,137] At any given timepoint, our IgG N-glycome is a heterogeneous mixture of various glycan structures present at a different percentage. The majority of these glycans are neutral (a-, mono- and digalactosylated), while about 15–25% are sialylated glycans.[17,34,134] Most of them are attached to the IgG Fc region, with about 15–20% of total N-glycans belonging to the Fab domains.[134] The differences in the composition of Fab and Fc glycans have already been described in Section 4. To add another layer of complexity, the Fc glycans of different IgG subclasses also vary in their glycopattern,[203] for reasons that are still not entirely clear.

6.2 Inter-individual variation of IgG glycome

On the population level, human IgG N-glycome is characterized by great variability in glycan abundances,[34,204] with galactosylation as the most variable of traits.[184,204] This is best illustrated by the level of the agalactosylated core-fucosylated N-glycan (FA2 in Table 1), which can comprise from roughly 6 to 50% of the total IgG N-glycome in healthy individuals.[34] In large-scale studies, inflammatory conditions and aging are typically characterized by a lower level of galactosylation and sialylation and sometimes an increased level of bisection in the group of diseased/aged compared to the group of healthy/younger people.[31,32,205] Differences in total, Fc, and (to a degree) Fab glycopattern between diseased and healthy population for a vast number of various pathologies have been extensively studied - reviewed in Refs. 31,134.

6.3 Intra-individual variation of IgG glycome

On an individual level, determining the relative abundance of a-, mono-, digalactosylated, and mono- and di-sialylated structures in principle allows for the determination of a person's general IgG glycoprofile type. Although the overall IgG glycoprofile (order of the most abundant structures) mostly remains stable,[34,206] there are physiological and pathological states that influence the abundances of certain structural groups of glycans - reviewed in[31] and discussed in detail in Section 8. For instance, galactosylation is known to change gradually with aging and the development of certain inflammatory conditions but can also shift rather abruptly following strong immune and physiological triggers, such as cardiac or bariatric surgery and sex hormone interventions.[40,43,206] The fact that the large inter-personal variation might mask the potentially non-healthy intra-personal variation emphasizes the importance of longitudinal follow-up to translate epidemiological research results into a clinical biomarker or a biomarker of biological age.

6.4 IgG glycosylation is affected by IgG amino acid sequence

The aforementioned complexity of the IgG glycome plasticity highlights one of the biggest unsolved problems of IgG glycosylation to date - its regulation. Perhaps the most straightforward reason for the variability of IgG glycome composition states that the IgG glycoprofile is associated with protein structure and steric balance within the molecule. In accordance with this proposition, IgG molecules bearing mutated CH2 amino acid residues at positions spatially close to the glycan in secondary or tertiary glycoprotein structures contain a changed glycosylation profile.[207–209] Likewise, IgG glycosylation patterns are reported to differ among subclasses,[203] although it is not at all clear to what degree this is structure- or function- related. The Fab glycoprofile also appears to be dependent on the positioning and amino acid surrounding of the glycosylation sites acquired through somatic hypermutation.[210–214] Additionally, the differences in N-glycan content between the Fab and Fc regions are presumably connected with the accessibility of the N-glycosylation sites to the glycosylation machinery of the Golgi compartment, as discussed in Section 4.

However, although well-founded, and correct to a degree, the structural proposition does not fully account for the occasionally striking differences of IgG glycan profile within an individual, most notably in case of various diseases, response to therapy, or lifestyle interventions.[40,41,43,215–221] It also does not explain the differences observed in the glycosylation of antigen-specific versus total IgG in infectious and autoimmune diseases and following vaccination.[222–225] In conclusion, although relevant, the amino acid sequence is surely not the only mechanism regulating the IgG glycome composition and is considered to contribute, at most, only moderately to the orchestration of the IgG glycan abundances.

6.5 IgG glycosylation is influenced by activity of glycosyltransferases

Another obvious explanation proposes that regulation takes place at the level of enzymatic machinery responsible for IgG glycan biosynthesis, such as various glycosyltransferases, glycosidases, and the availability of activated nucleotide sugar donors.[226] Of those, the most explored are variations in the IgG glycoprofile in association with the change in the expression or activity level of the main glycosyltransferases participating in the extension of the glycan core to form complex IgG N-glycans: fucosyltransferase 8 (FUT8, responsible for the addition of core-fucose), *N*-acetylglucosaminyltransferase 3 (MGAT3, responsible for the addition of bisecting GlcNAc), β-1,4-galactosyltransferase 1 (B4GALT1, responsible for the addition of galactose), and α-2,6-sialyltransferase 1

(ST6GAL1, responsible for the addition of sialic acid).[227] Indeed, a decreased level of IgG galactosylation was associated with reduced activity of B4GALT1 in peripheral B cells of RA patients,[228–231] as well as in myeloma cells.[232] However, other studies found no difference in the expression of B4GALT1 in RA patients compared to healthy controls.[233,234] In a study on postmenopausal female mice, estrogen-induced increase in IgG Fc sialylation was also accompanied by an increased expression of ST6GALT1 in splenic plasmablasts,[42] thus linking the expression of ST6GALT1 with the sialylation of IgG, as was previously reported in RA patients.[235] The association of ST6GALT1 expression and the level of sialylated antigen-specific IgG was also confirmed in mice immunized with ovalbumin (a T cell-dependent antigen) under both inflammatory and tolerogenic conditions.[236]

Although glycosyltransferase activity is undoubtedly of key importance in some instances, the lack of consistency in expression data indicates that the glycosyltransferase transcriptome of antibody-producing cells generally remains a poor predictor of their IgG glycome, as is often the case with other proteins.[237]

Of note, recent evidence suggests that IgG glycans can be modified extracellularly post-secretion by glycosyltransferases present in the bloodstream, although the degree of this change is still debated.[15,16,235,238,239]

6.6 IgG glycosylation is influenced by genes

Protein glycosylation is a complex sequential co- and post-translational process that, in contrast to protein synthesis, occurs without a direct genetic template.[240] It is mediated by glycosyltransferases, glycosidases, nucleotide sugar transporters, and other proteins directly involved in the glycan biosynthesis and attachment to the nascent proteins, but also transcription factors, ion channels, glycan-binding proteins, enzymes, and other proteins that affect glycosylation indirectly – by influencing cell metabolism, particularly the activity of endoplasmic reticulum and the Golgi apparatus.[14,241] Indeed, as many as 5% of all genes are considered to code for proteins directly or indirectly involved in protein glycosylation.[237,241] Genome-wide association studies (GWAS) have found that IgG glycosylation is regulated through large genetic networks that are pleiotropic with hematological cancers and autoimmune and inflammatory diseases.[242–244] However, the importance of genetic regulation of IgG glycosylation is best reflected in the results of heritability studies. They found that the genetic component explains for a significant percentage of variance in IgG glycan abundance - up to 48% in one, and up to 76% in the other study, depending on the glycan structure.[33,34]

6.7 IgG glycosylation is influenced by environment

In response to various stimuli, the glycome is in general more dynamic than the transcriptome and the proteome,[237] and the IgG glycome is no exception. Indeed, the IgG glycome is modified in response to sex hormone intervention,[40,42] as well as in response to therapy for infectious, autoimmune diseases and cancer.[215,220,221,245–248] In addition, the IgG glycosylation pattern is associated with environmental factors pertaining to lifestyle, such as smoking, dietary supplements, and exercise.[38,39,41,249] It is suggested that the environmental control of IgG glycosylation processes acts through the modulation of gene expression by epigenetic mechanisms in B cell and other relevant cell lines.[33,249–251]

On the level of cellular environment, the IgG glycome is affected by the metabolic and immune context of T- and B-cell activation and IgG production.[235,236,252–259] A difference in antigen nature (polypeptide versus polysaccharide), i.e. the need for T-cell help in mounting the immune response, and immunization protocols seem to result in a composition of IgG glycoforms distinctly different from the one of total serum IgG.[260,261] Likewise, various metabolites and molecules involved in the immune activation, such as retinoic acid, CpG oligonucleotide or interleukin-21, stimulate the B-cells to produce differentially glycosylated IgG *in vitro*.[256]

6.8 Integration of all regulatory determinants at the level of IgG glycan biosynthesis

In conclusion, the pattern of IgG glycosylation seems to be regulated on several levels: protein structure stemming from differences in primary amino acid sequence; intracellular localization and the milieu regulating the enzymatic reactions – such as level of enzymes and substrates, but also more subtle factors, including, e.g. pH and ionic strength; tissue environment and immune context – such as inflammatory signals and the mode of immune activation; and "out-of-body" environmental factors – such as antigen exposure, and medical and lifestyle interventions (Fig. 4). Interestingly, the change in the IgG glycosylation pattern can be extensive and rapid (e.g. in acute inflammatory diseases and extreme weight loss[43,206]), but also slow and gradual (e.g. during aging[36]).

Different levels of IgG glycosylation regulation are interconnected, and the signals are relayed in both directions: "top-down" – when the experiences of the whole organism are brought down to cellular level and influence gene expression through epigenetic and transcription mechanisms; and "bottom-up" – when gene variants influence the glycan biosynthesis machinery. Finally, glycans likely serve as active modulators of IgG function, affecting the homeostasis or response on the level of tissue or whole organism. Thus, IgG glycans not only reflect the intricate interplay between genes and environment,[250] but probably also play an active role in homeostasis maintenance and immune responses. The precise mechanistic explanation for most of these processes is still lacking.

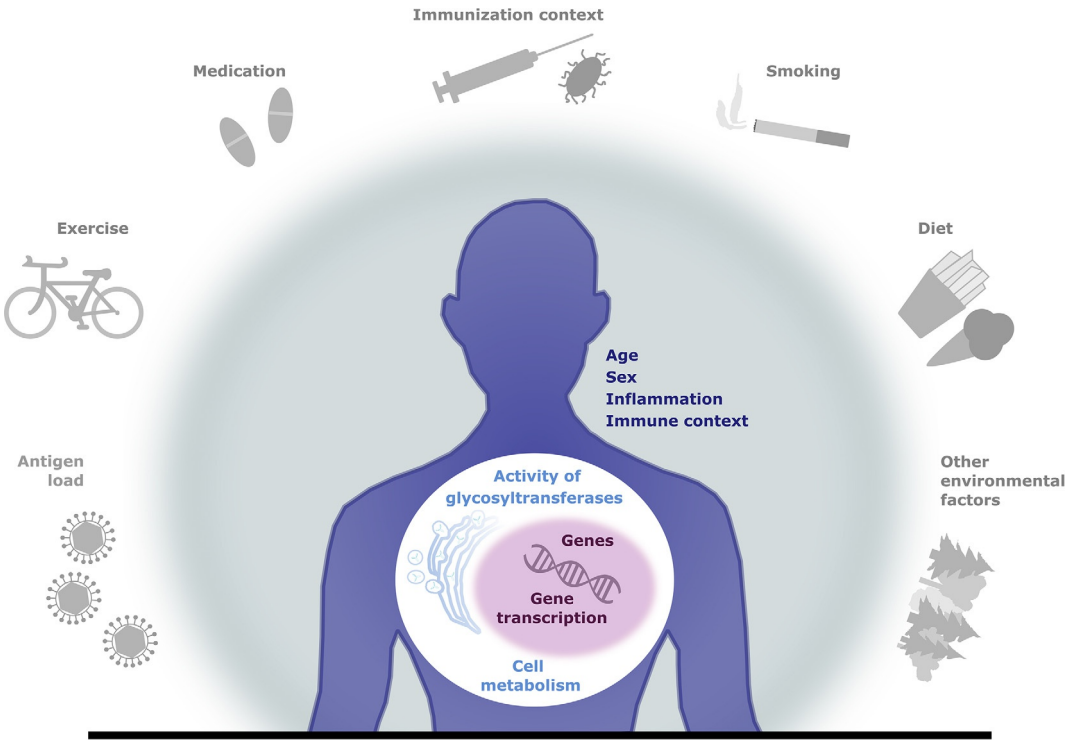


Fig. 4 Complex regulation of immunoglobulin G (IgG) glycosylation. IgG glycosylation is regulated on several interconnected levels, from genetic variants and immune context to environmental factors.

6.9 Open questions

The remaining open questions concerning the regulation of IgG glycosylation are: Is the specific change of IgG glycopattern achieved through the expansion of B cell clones with the targeted IgG glycoprofile? Or is a specific glycopattern a consequence of the cellular and tissue environment and not fixed at a cellular level? Is a particular pattern of IgG glycosylation selected for during affinity maturation? Is it defined (and, if so – how) during the process of clonal expansion of B cells upon immune activation? Does this putative IgG glycopattern fixation pertain in the long-lived plasma cells, and survive throughout the memory B cell formation and reactivation upon repeated antigen challenge? How are antigen-specific antibodies directed towards asymmetrical Fab glycosylation?

Hopefully, coming closer to resolving the daunting complexity of the IgG glycosylation regulation will also shed light on the still non-resolved question of the possible targeted functionality of the acquired IgG glycopatterns.

7 Glycan composition modulates IgG effector functions

The structural composition of the Fc-bound glycans has an effect on functional properties and activity of IgG due to structural changes in the regions of the molecule that interact with its receptors and ligands: the CH2 domain and the CH2-CH3 domain interface (Fig. 1).[116,262–265] Even though the IgG glycosylation profile should be observed in its entirety for comprehensive understanding, here we will first focus on the known effects of the isolated IgG glycosylation traits (fucosylation, bisection, galactosylation, sialylation) on interactions of IgG and the components of the immune system (Fig. 5). The diseases characterized by any of the vast arrays of changes in the IgG glycoprofile have been extensively reviewed recently and will not be listed here.[31]

7.1 Fc fucosylation

Over 90% of all Fc glycans carry a core-fucose attached to the innermost GlcNAc of the glycan core structure.[24,134,203] This is in stark contrast with the majority of other plasma proteins, which are not core-fucosylated.[45] The absence of core fucose significantly increases the affinity of IgG for the FcγRIIIA and FcγRIIIB, enhancing the downstream effector functions, most notably the NK cell-mediated ADCC, up to 100-fold.[263,266,267] Interestingly, the N-glycan bound to the Asn-162 of the receptor itself proved crucial for the high-avidity interactions between the afucosylated IgG and the FcγRIIIA.[268] The enhancement of ADCC for afucosylated

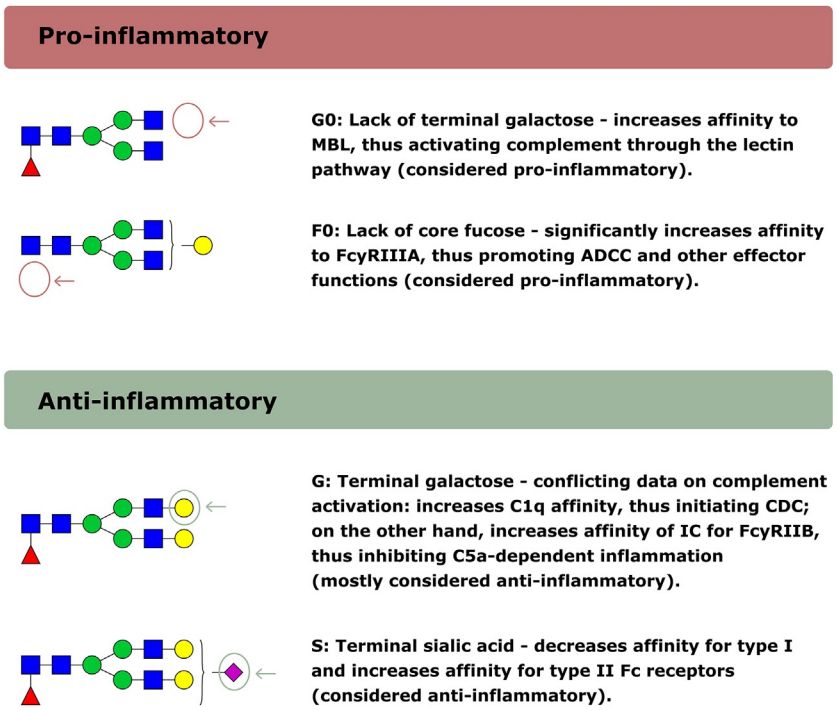


Fig. 5 Differential immunoglobulin G glycosylation modulates its effector functions. Representatives of certain glycan structural groups and their most notable molecular interactions are depicted. ADCC = antibody-dependent cellular cytotoxicity, CDC = complement-dependent cytotoxicity, FcγR = Fcγ receptor, MBL = mannose-binding lectin.

antibodies is the most striking effect of glycan structural variation on the IgG function, and it is heavily employed in the production of mAbs that rely on this mechanism.[269]

Core-fucosylation seems to be irrelevant for complement activation by binding to C1q.[263]

7.2 Fc bisecting GlcNAc

Only about 10% of all IgG Fc glycans contain a bisecting GlcNAc.[134] The presence of bisecting GlcNAc is sometimes associated with enhanced binding affinity for FcγRIIIA and B, and consequently, the related downstream effector functions.[270–272] However, since the addition of the bisecting GlcNAc and core fucose have a partially mutually excluding effect on the level of glycan synthesis,[273–275] it is sometimes difficult to distinguish the functional roles of the absence of core fucose from the presence of bisecting GlcNAc and vice versa.[267] A more recent study reported no significant effect of bisecting GlcNAc on binding any of the FcγRs or C1q.[263]

7.3 Fc galactosylation

In healthy adults, on average, 35% of IgG Fc glycans are agalactosylated, another 35% carry one, and about 15% two terminal galactoses.[199,203]

7.3.1 Type I FcγRs and complement

Terminal galactose residues affect IgG inflammatory potential by modulating binding affinities to downstream effector molecules, namely complement components and FcγRs. Fc glycans lacking terminal galactoses are proposed to act pro-inflammatory by activating the complement both through the alternative pathway,[276] and through the lectin pathway the following binding to the mannose-binding lectin (MBL).[122,277,278] Likewise, terminal galactosylation was found responsible for the anti-inflammatory activity of immune complexes through binding to the inhibitory FcγRIIB followed by the inhibition of the C5a-dependent inflammation.[279] On the other hand, Fc galactosylation is known to enhance CDC through the classical pathway of complement activation by increasing the affinity of IgG for C1q complement component,[147,280,281] and to enhance FcγR-mediated processes, particularly ADCC, by increasing the IgG affinity for FcγRs.[263,282–285] Thus, although IgG galactosylation is often considered simply "anti-inflammatory," the reality is, as usual, way more complex and context-dependent.

7.3.2 FcRn

Although galactosylation had previously been considered irrelevant for the IgG's affinity for FcRn,[149] a more recent study found that the increased affinity of galactosylated IgG glycans for FcRn resulted in a selective placental transfer of NK cell-activating antibodies from mother to the fetus, thus arming the neonati to mount a better innate immune response.[148]

7.3.3 Fc structure

The precise mechanistic explanations for the change of affinities towards the FcRn and FcγRs for differentially galactosylated Fc glycans are still missing. However, there are reports that terminal galactose of the α1,6 arm additionally anchors that arm to the polypeptide backbone.[286] This would suggest that the variation in the Fc glycan composition influences glycan mobility as a manner to fine-tune the Fc conformation, which translates into a changed affinity for receptor molecules.[133]

7.4 Fc sialylation

In healthy adults, on average, 10–15% of IgG Fc glycans are either mono- or disialylated.[199,203] Terminal Fc sialic acids are most often considered in the context of modulation of the IgG inflammatory capacity.[287]

7.4.1 Fc structure

The effects of Fc sialylation on the Fc domain conformation are, alongside core fucosylation, probably the most explored among all Fc glycan traits. The Fc domain is found to alternate between the "open" and "closed" conformation, depending on the sialylation status of the Fc glycan: when terminal sialic acids are absent, the "open" conformation favors binding to the type I FcγRs close to the hinge-proximal surface of the CH2 domain.[133] In contrast, in the presence of a sialylated glycan, the Fc region gains additional flexibility and adopts the "closed" conformation, which favors binding of type II FcRs at the revealed binding site at the CH2-CH3 domain interface.[133,288] Molecular modeling suggests that the adaptation of the closed conformation is a result of the rotation of the Phe-241 away from the glycan – this allows for greater mobility of the α1,3 glycan arm, which moves out of the internal cavity.[133] Thus, the capacity of the Fc domain to alternate between two conformational states, depending on its sialylation status, is suggested to serve as a means to switch between two distinct receptor specificities, resulting in opposing immunological outcomes. However, it is important to say this was not confirmed in a different study.[289]

7.4.2 Type I FcγRs

Reports on the consequences of Fc sialylation on the binding of type I FcγRs are somewhat ambiguous: the effect seems to be either non-existent or slightly decreasing the affinity for type I FcγRs binding.[263,285,290,291] Studies on the magnitude of the inflammatory response and FcγRIIIA-mediated ADCC by NK cells consistently report conflicting results.[178,209,263,285,290,292,293]

7.4.3 Type II FcRs and IVIg mode of action

The preferential binding of the Fc containing sialic acid to the C-type lectin receptors became evident when the importance of the sialylated Fc fraction for the anti-inflammatory activity of the IVIg preparation was established in a K/BxN serum-transfer mouse model of RA.[178] Mouse in vivo studies on various antibody-dependent autoimmune disease models have confirmed that the sialylated Fc fraction binds to specific ICAM-3 grabbing non-integrin-related 1 (SIGN–R1), the mouse orthologue of the human dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN), on the surface of splenic macrophages. Receptor activation induces IL-33 secretion, which in turn leads to the release of IL-4 by basophils, followed by enhanced FcγRIIB expression on the effector macrophages.[18,178,294–300] However, this finding did not translate to human studies and was even conflicted in other *in vitro* and in vivo models[297,301–304] pointing to the fact that there is more than one effector mechanism behind the IVIg mode of action, and its effectiveness is highly dependent on the disease and immune context.

7.4.4 Complement C1q

The reports on the effect of sialylation on C1q binding and CDC induced by the classical pathway are conflicting,[263,291] calling for more research on the topic.

7.5 Fab glycosylation

7.5.1 Antigen binding

Modulation of IgG effector functions in cases where the absence of Fab glycans or their differential composition affect antigen binding[157,211,305–312] is self-evident and thought to arise from the conformational changes in variable domains inferred by glycan presence or composition. However, the fact that the antibody activity can change in the absence of the Fab glycan without an effect on antigen binding indicates that other steric effects can be induced by the presence of glycans in the Fab region.[313]

7.5.2 Immune complex formation

As described in Section 4.7, in cases where the presence of the asymmetrical Fab glycan inhibits antigen binding in only one of the paratopes, IgGs that are asymmetrically glycosylated in the Fab region are functionally univalent and unable to form large immune

complexes.[169] This modification can be both positive or negative, depending on the biological setting and the original immunogen: harmful pathogen antigens, non-harmful self-antigens, or allergens. The synthesis of IgG with asymmetrically glycosylated IgG is suggested to intensify during the T_H2-skewed immune response, such as in chronic infections by extracellular microorganisms, pregnancy and allergies, where they act beneficial to the host by exerting regulatory functions.[169]

7.5.3 Aggregation and precipitation

The composition of Fab glycans has been reported to modulate the IgG tendency for aggregation and precipitation.[314] Interestingly, this is also shown for IgG cryoglobulins – immunoglobulins that reversibly precipitate at reduced body temperatures and are associated with various infectious, malignant and autoimmune diseases.[315] The presence of sialic acid on cryoglobulins was found responsible for their unusual solubility at a low temperature.[316]

7.5.4 IVIg mode of action

As described above, sialic acid residues attached to the Fc glycans were initially the ones considered responsible for the anti-inflammatory activity of IVIg preparations in autoimmune diseases.[178,294] However, a number of studies suggest that it also depends on its sialylated Fab fraction, at least in part.[317–321] Whether the downstream effects occur through the binding of sialylated Fab glycans by one of the lectins of the immune system, as is the case with sialylated Fc glycans, such as DC-SIGN, DCIR, and CD22,[319–322] or through conformational changes of the IgG induced by the sialylated Fab glycans is a matter of discussion.[323]

8 Changes in IgG N-glycosylation associated with physiological states

8.1 Aging

8.1.1 IgG galactosylation

The first study that observed an association between the IgG N-glycosylation profile and age was published as early as 1988.[23] Parekh et al. observed an association of IgG glycopattern, namely the galactosylation level, with age: the abundance of galactosylated IgG reached a peak at about 25 years of age and then decreased with the advancing age.[23] Since then, galactosylation remains the most explored IgG glycosylation trait in relation to aging, but also in many pathological states as well.[31,205] Numerous research studies conducted in the years following the first paper confirmed the original finding and reported that early adulthood is indeed characterized by a similar level of digalactosylated and agalactosylated IgG glycans, and an increasing ratio of agalactosylated to digalactosylated glycans with age.[196,324–328] This was found true on the level of released (Fab + Fc) glycans and on the level of subclass specific Fc glycans (analyzed as Fc glycopeptides),[196,324–328] and confirmed by large-scale studies consisting of up to several thousand subjects across multiple populations.[34–36,203,329–331] The reports on the association of the abundance of monogalactosylated IgG glycans with age are inconsistent.[205]

Presumably due to more stringent ethical constraints, the studies on children were typically performed on smaller populations.[205] When it comes to galactosylation, some (but not all) studies reported a trend opposite to the one observed in adult populations – namely an increasing abundance of digalactosylated and a decreasing abundance of agalactosylated IgG glycans with age.[23,187,330] Notably, in our study, which comprised by far the largest number of children (over 600), this effect was only observed on IgG4,[332] while other studies reported this for males/females only,[326,333] or reported no change in IgG galactosylation level during childhood and adolescence.[328] The inconsistency between pediatric populations likely originates in lower sample sets (compared to adult populations), study design as well as a physiologically turbulent period observed.[205]

Since most of the a-, mono- and digalactosylated N-glycans released on the level of total plasma/serum proteome actually come from IgG,[45] total plasma/serum N-glycome can be used as a proxy for evaluation of the neutral portion of the IgG N-glycome.[334]

8.1.2 Inflammaging

Balancing the pro- and anti-inflammatory responses is crucial for successful aging – a period of life not accounted for by evolutionary selection mechanisms. During a lifetime, every human being is exposed to a variety of external (bacteria, viruses, toxins, etc.) and internal (e.g. damaged tissues and cells) stimuli that trigger a response from the built-in defense systems in the form of inflammation. With aging, prolonged exposure to pathogen-associated molecular patterns (PAMPs) and endogenous danger associated patterns (DAMPs) causes an imbalance between pro-inflammatory networks designed to fight the recognized danger and the anti-inflammatory networks designed to keep the homeostasis.[335] The long-lasting inflammation, initiated with the intention to protect the organism during a life-time of antigen load and immune triggering, results in a pro-inflammatory remodeling of the immune system: a low-grade chronic sterile inflammatory state characteristic for the elderly.[336–338]

It is proposed that the age-related gradual decrease in the level of galactosylated IgG and the build-up of agalactosylated IgG is not only one of the hallmarks of this chronic low-grade inflammation but also exacerbates it (Fig. 6).[337–339] Thus, in a vicious self-fueling loop, agalactosylated IgG acts as both (1) a biomarker of aging and (2) an effector of its pro-inflammatory pathological changes.[334,335] Decreased inflammation has indeed been proven a good predictor of healthy aging,[340] thus establishing the association between inflammaging levels and the risk of age-related diseases.[336,341]

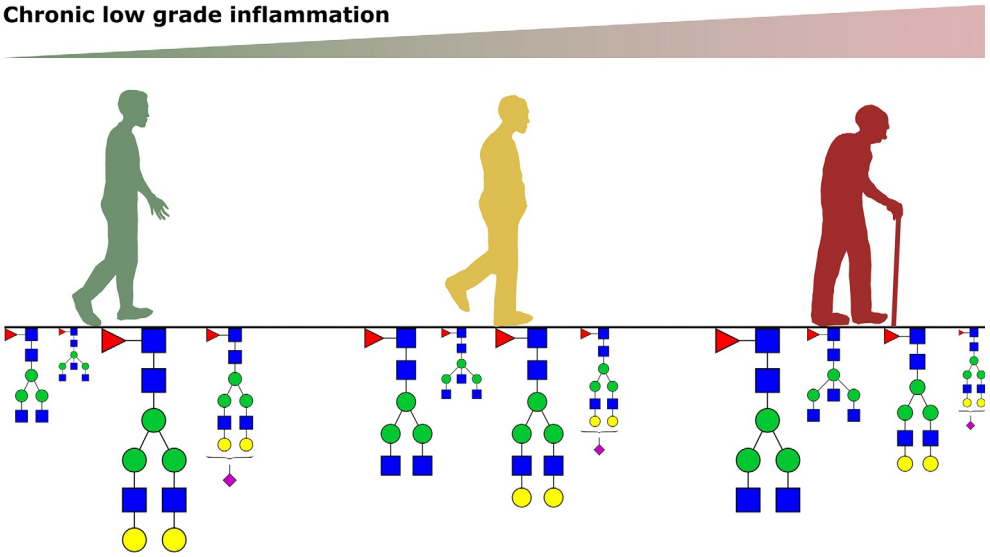


Fig. 6 Dynamics of immunoglobulin G (IgG) glycome composition with age. From young adulthood to old age, the abundances of galactosylated and sialylated glycans decrease, while the abundance of glycans containing bisecting *N*-acetylglucosamine increases. The inflammaging hypothesis proposes that this age-related accumulation of pro-inflammatory IgG glycoforms in turn acts as one of the effector mechanisms of the aging process, enhancing inflammation in a vicious self-fueling loop. Representatives of certain glycan structural groups are depicted.

8.1.3 IgG sialylation

The seminal paper by Parekh et al. found no association of the abundance of sialylated IgG with age in the observed adult population.[23] Later studies on adults found the level of sialylation to decrease with age in specific subsets: a particular glycan trait, an age group or an IgG subclass.[196,325,327] Large-scale studies mostly agree in reporting an age-associated decrease in the abundance of sialylated IgG glycans, which is probably at least partly connected to the same dynamics observed for the level of galactosylated IgG glycans.[34–36,203,331] When it comes to the abundance dynamics in childhood, the level of Fc sialylation was found to decrease by the age of 10, after which age the reports are inconsistent.[187,328,330,332]

8.1.4 IgG bisection

The dynamics of the level of bisected IgG glycans was only looked into about 10 years after the study by Parekh et al.[23,326] The level of IgG glycans containing bisecting GlcNAc was found to increase in association with increasing age, as confirmed by other smaller[196,326,327] and large-scale studies.[34,203,330] Studies performed on pediatric populations mostly found that the abundance of glycans containing bisecting GlcNAc was increasing throughout the young age.[205]

8.1.5 IgG core fucosylation

The reports on the dynamics of the abundance of core-fucosylated IgG glycans in adult populations are conflicting, ranging from no change to a mild increase or decrease in abundance.[205] The studies on children, however, consistently report a decrease in the abundance of core fucosylated structures in association with age.[187,328,330,332]

Indeed, the strong association of IgG glycome composition and age is confirmed by the fact that the abundances of IgG glycans alone explain up to 65% of the variation in chronological age.[35,36]

8.2 Sex hormones

8.2.1 Sex

Besides age, sex hormones are likely the second most important physiological factor affecting IgG glycosylation. Many studies investigating associations of IgG glycosylation and aging (listed above) also observed differences between male and female adults. They often report higher levels of agalactosylated IgG glycans in males than females until the age of menopause, when the abundance of galactosylated glycans drops more sharply in females. After menopause, the level of galactosylated IgG is lower in females than males and decreases gradually at a similar pace in both sexes.[36,203,324,326,329,330] The notable drop of the galactosylation level associated with the period of menopausal transition is accompanied by the drop of sialylated IgG glycans.[203,330] The differences in the IgG glycopattern between the two sexes are much less prominent in the pediatric populations – if at all present, they are inconsistent and only take place around the onset of puberty.[23,187,328,330,332,333]

8.2.2 Pregnancy and menstrual cycle

The association of IgG glycosylation pattern and female sex hormones level was also observed during pregnancy, a period characterized by increased levels of Fc galactosylation and sialylation.[195,217,342,343] IgG glycosylation measured at three time-points during pregnancy (once per trimester) showed an increase of galactosylation, sialylation, and the number of sialic acids per galactose in the transition between 1st and 2nd trimester when the levels of human chorionic gonadotropin (hCG) increase most rapidly, while the changes observed in the period *post partum* were most significant between 0 and 6 weeks postpartum when the levels of estrogen and progesterone rapidly decrease.[195] Interestingly, increased pregnancy-related galactosylation was associated with a decrease in disease activity in RA patients, hinting at the functional role of agalactosylated Fc in disease pathogenesis.[217,342,344] The single study looking into the pregnancy-related glycosylation pattern of Fab and Fc fragments separately reported a similar pattern for Fab and Fc. Namely, in addition to the increased galactosylation and sialylation and decreased bisection of the Fc portion increased sialylation and decreased bisection was found on the Fab portion, while the galactosylation level of Fab glycans is probably around the physiological maximum even in non-pregnant females, so it can hardly increase.[343] It is suggested that the observed changes in glycopattern both on Fab and Fc might reflect the pregnancy-induced immune suppression.

Variations of abundances of different IgG glycosylation traits in association with the fluctuating levels of sex hormones are also observed in the single study looking into the IgG glycopattern dynamics in the menstrual cycle.[345]

8.2.3 Interventional studies

Although associations of sex hormones and IgG glycosylation were published repeatedly, it was the interventional studies that proved the causal relationship between the two phenomena: an impressive study utilizing endocrine manipulation in postmenopausal women, premenopausal women, and men revealed estrogen as a modulator of total IgG galactosylation in both sexes,[40] while the repeated analyses of the same samples extended to the effect on sialylation.[346] Another recent study confirmed that estrogen supplementation increased IgG sialylation in post-menopausal mice and female RA patients, probably by inducing ST6GAL1 expression,[42] offering a potential mechanistic explanation for the increased risk for RA in post-menopausal women. Moreover, these very exciting results represent one of the rare examples of successful manipulation of IgG glycosylation profile, possibly leading the way towards the development of therapeutics that target IgG glycosylation as one of the underlying functional mechanisms of pathological processes.

9 Changes in IgG N-glycosylation associated with diseases

The first study that looked at the association of a disease with IgG glycosylation changes was published in 1985.[22] The authors reported a higher level of agalactosylated IgG glycans in RA patients compared to healthy controls.[22] This finding was later confirmed by many others,[347–352] and to date RA remains the most investigated disease from the aspect of IgG glycosylation (described in more detail in Section 9.1). Since then, a changed IgG glycoprofile has been associated with a plethora of auto- and alloimmune, infectious, cardiometabolic, malignant, and other diseases.[31]

Since the initial study, scientists have been puzzled by the role of these changes, and today there is still no definite answer. The fact that IgG glycosylation changes can precede the disease by many years, e.g. in RA,[225,353,354] suggests that IgG glycans reflect a predisposition, or, in the case of antigen-specific IgG,[225,355] more likely an effector in disease pathogenesis. Multiple glycan traits associated with a vast array of heterogeneous diseases probably indicate that it is not one but several different pathways that are interconnected with IgG glycosylation and could be involved in disease occurrence and progression. However, the overall situation is, as usual, extremely complex and depends on the disease and context, with no general rule that can be applied. It is very likely that, depending on the disease and IgG subset, IgG glycoprofile different from the one found in healthy population can represent a predisposition, direct molecular effector, or a non-functional "side-effect" reflecting the immunological context of an individual.

9.1 Autoimmune and alloimmune diseases

Since the seminal paper on RA,[22] today we know that the decreased galactosylation of bulk IgG and Fc not only accompany RA, but they are also associated with the disease progression and activity, as well as with the response to therapy.[217,342,344,347–352,356] Moreover, a low level of IgG galactosylation is associated with an increased risk of the future RA diagnosis and adverse disease outcome in patients with undifferentiated arthritis.[353,357] Anti-citrullinated protein antibodies (ACPA), which are involved in RA development, show a decreased level of galactosylation and sialylation compared to bulk IgG, not only in association with, but also preceding the clinical onset of the disease.[225,344,354,358] Lately, the glycoresearch in RA has focused on the Fab portion of ACPA. Perhaps the most interesting observation comes from recent studies reporting an increased amount of Fab-linked glycans,[359,360] which are highly sialylated.[360] This increase is absent from ACPA$^+$ healthy subjects,[361,362] implying that ACPA Fab glycosylation might be the molecular effector leading to the breach of tolerance, possibly by modulation of antigen binding.[359]

Although the most explored, RA is not the only autoimmune disease with reported changes in IgG N-glycosylation profile.[243] Decreased levels of bulk and/or antigen-specific IgG galactosylation and/or sialylation have been associated with a wide range of other autoimmune conditions, such as systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis and many others. Changes in these IgG glycosylation traits are also associated

with disease progression, disease activity, symptom severity, and response to treatment. Moreover, occasionally these changes occur before the onset of symptoms (extensively reviewed in[31]).

Two alloimmune diseases, fetal and neonatal alloimmune thrombocytopenia (FNAIT) and hemolytic disease of the fetus and newborn (HDFN), are characterized by a decreased level of core fucosylation on the Fc of antigen-specific IgG.[363–367] Since these are antibody-mediated diseases, and the level of core fucosylation on the corresponding antigen-specific IgG is associated with disease severity and risk for serious clinical outcome, IgG glycosylation is, in this case, most likely one of the effector mechanisms that contribute to disease pathology through FcγRIIIA-mediated ADCC.[363–366]

There is no single one-size-fits-all explanation for the role of IgG glycans in the auto- and allo-immune diseases. Shared loci associated with both autoimmune diseases and IgG glycome composition[242] and the fact that IgG glycome pattern can change prior to disease development but independent of time to diagnosis[353] imply that the changes observed on bulk IgG likely reflect a disease predisposition. This is a trait most likely shared between all diseases with an inflammatory component where the pro-inflammatory glycoprofile of total IgG probably plays a role in modulation of immune activation threshold. In RA, agalactosylated IgG is additionally suggested to have a higher affinity for rheumatoid factor and is thus responsible for the enhanced formation of autoantibody aggregates.[368] However, changes on antigen-specific IgG, particularly in antibody-dependent auto- and alloimmune disease, combined with the modulation of downstream effector functions (as is the case in HDFN and FNAIT),[363–366] support the notion about their functional role. Thus, the role of IgG glycans in various auto- and alloimmune diseases varies according to the particularities of the disease pathology.

9.2 Malignant diseases

The first report on a skewed IgG glycosylation pattern in a malignancy showed a high level of agalactosylated IgG in multiple myeloma patients, accompanied by decreased activity of B4GALT1 in their bone marrow cells.[232] Since then, somewhat conflicting results have been observed for glycosylation traits of IgG from myeloma patients.[248,369,370] Similarly, there is no general rule for the change in IgG glycosylation pattern in patients suffering from different cancers, although, in addition to multiple myeloma, quite a few of them show a decrease in the abundance of galactosylated IgG compared to healthy controls: Ovarian cancer, prostate cancer, non-small cell lung cancer, gastric cancer, lung cancer, breast cancer and colorectal cancer (reviewed in[31]).

This decrease of IgG galactosylation has been suggested to be either reflective of the host's defensive inflammatory response to cancer,[371] acute-phase response pathways that are involved in cancer progression,[372] or impaired binding of IgG Fc to C1q complement component, resulting in reduced CDC and cancer cell escape.[373]

Several types of malignant diseases (e.g. B cell malignancies) show an enhanced N-glycosylation in the Fab region, often characterized by high-mannose structures.[156,374–377] The newly acquired glycans are suggested to contribute to disease pathogenesis by enhancing tumor cell persistence and expansion, possibly by binding to lectins on the surface of innate immune cell types, such as dendritic cells and macrophages, and thus liberating tumor cells from dependence on antigen binding.[375,378]

9.3 Infectious diseases

The majority of infectious diseases that have been studied from the aspect of total IgG glycosylation report a lower level of galactosylation (and, in some cases, sialylation) in diseased compared to healthy subjects, as evidenced by the IgG glycopattern in patients suffering from tuberculosis,[246,379–381] HIV infection,[382,383] hepatitis B and C[245,384] and others – reviewed in Ref. 31. Moreover, in hepatitis B the abundance of galactosylated IgG was associated with disease severity and reported to revert to the initial levels in response to therapy.[245] The latter is also true for tuberculosis.[246] Very timely, recent studies on COVID-19 report that patients suffering from a severe form of diseases have lower IgG galactosylation and sialylation levels (as well as lower bisection levels) compared to patients suffering from a mild form of the disease.[223,385] The glycosylation pattern of total IgG in these cases is most likely associated with a general pro-inflammatory status upon infection.

In contrast to auto- and allo-immune diseases, in infectious diseases, antigen-specific IgG plays the role it was intended for by the forces of evolution – protection against pathogenic antigens. Contrary to the bulk IgG, changes in its glycosylation pattern are probably associated with the IgG functionality. For instance, increased binding of afucosylated dengue virus-specific IgG to the FcγRIIIA is considered responsible, through platelet depletion, for the antibody-dependent enhancement - a mechanism at the core of severe dengue hemorrhagic fever and dengue shock syndrome occurring upon secondary dengue infection.[224] Similarly, antibodies targeted against SARS-CoV-2 exhibit a higher level of core-fucosylation in critically ill patients compared to patients who clear the infection unaided.[223,386] Moreover, afucosylated anti-Spike antibodies are proposed to mediate increased pro-inflammatory cytokine production by primary monocytes and *in vitro* generated lung macrophages, resulting in a disruption of pulmonary endothelial barrier, and microvascular thrombosis – hallmarks of the severe COVID-19.[386,387] Interestingly, spontaneous HIV controllers exhibit, in addition to a general increase of agalactosylated bulk IgG, a more profoundly skewed gp120-specific IgG glycoprofile.[382] This profile is characterized as highly pro-inflammatory (with decreased abundances of core-fucosylated, galactosylated, and sialylated glycan species), pointing at a two-level regulation of IgG glycosylation in infectious diseases: general inflammatory cues for bulk IgG, and effector function-associated fine-tuning for antigen-specific IgG.[382] This emphasizes the fact that IgG glycan composition should be interpreted in the context of the disease-characteristic pathological mechanisms. Moreover, the distinct glycoprofile of IgG specific for the influenza envelope (characterized by increased galactosylation and sialylation) and the lack of correlation between glycoprofiles of IgG directed against different antigens from the same

individuals suggests the skewing of IgG glycopattern is disease-specific, and independent of the host genetic or immunological background.[388]

9.4 Cardiometabolic diseases

Cardiometabolic diseases, such as cardiovascular diseases (CVD), diabetes, and chronic kidney disease (CKD) are complex traits, their etiology based on interactions of multiple environmental and genetic factors.[389] Since they are also low-grade inflammatory disorders, it comes as no surprise that diseased and prediseased states, as well as disease risk factors, are associated with a changed IgG glycoprofile compared to a healthy population.[31]

Hypertension, a well-established risk factor for the development of CVD, is associated with decreased IgG galactosylation, sialylation and bisection accompanied with increased core-fucosylation.[390,391] Moreover, a study on over to 4700 subjects from two independent cohorts reported an association of decreased IgG galactosylation and sialylation with the 10-year cardiovascular disease risk score.[27] Very exciting discovery originated from a mouse study investigating the link between obesity and the development of hypertension.[38] IgG from mice in which obesity was induced by a high-fat diet (HFD) was hyposialylated compared to control mice, and caused a rise in blood pressure when transferred to IgG-deficient mice, thus demonstrating the functional role of IgG in the development of hypertension.[38] Furthermore, in HFD-fed mice supplemented with a sialic acid precursor, *N*-acetyl-D-mannosamine (ManNAc), IgG sialylation was restored and protected the mice from the obesity-induced hypertension development.[38]

A study conducted on more than 5000 subjects found that type 2 diabetes was associated with a decrease in IgG galactosylation and sialylation and an increase in the level of bisected IgG glycoforms.[26] The same IgG glycosylation pattern was associated with chronic kidney disease, a common, age-related complication of hypertension and diabetes.[392] In a recent study, the IgG galactosylation level was inversely associated with kidney failure, as measured by a decrease of estimated glomerular filtration rate (eGFR), during a 7-year follow-up.[25]

Unlike for hypertension, currently there are no studies confirming a functional role of the skewed IgG glycoprofile in diabetes and chronic kidney disease, so it is considered to reflect the overall inflammatory state characteristic for these diseases.

10 IgG as a biomarker of biological age

Aging is a natural process of micro- and macromolecular damage accumulation that affects individual phenotype and significantly impacts the fitness of the organism.

10.1 Biological age

It is well established that different people age at a different pace, which is often not in accordance with their chronological age. In contrast to chronological age, which is based exclusively on the amount of time that has elapsed since birth, biological age refers to health and functionality.[393] Biological aging is the process of sequential error accumulation and deterioration that occurs as a combination of genetic background, environmental stressors, and the passage of time. Throughout the years, many different aspects of biological aging have been investigated, including gene polymorphism, DNA methylation, telomere length, gene expression, metabolic health, oxidative stress, and IgG glycosylation.[394–396]

10.2 Biological age predictors

In the context of modern civilization where life expectancy is in many countries extended to 70+ years,[397] there is a rising interest for prolonging youth, retaining health, and increasing the quality of life, which includes preventing diseases or managing their severity. To achieve these goals, reliable predictors or markers of biological age are a necessity.

Although an intuitive explanation would claim a marker of biological age should enable the identification of individuals at risk of age-related conditions, disease, and death, there are multiple requirements that a successful biomarker of biological aging must satisfy. It is suggested that biomarkers of biological age should: (1) predict the rate of aging, i.e. denote where the person is in their total lifespan, better than chronological age, (2) predict the years of remaining good function, and the trajectory toward organ-specific illness in the individual, (3) be based on the processes that underlie aging, not its effects, (4) link the difference from chronological age to the existing markers of (un)healthy lifestyle, (5) be responsive to interventions that act beneficially on the biology of aging, (6) be minimally invasive in order to be repeatable without harming, (7) be accessible to masses, and (8) work in laboratory animals to enable extensive testing before validation in humans.[36,398–400] Historically, the two most-explored biomarkers of aging are telomere length and DNA methylation, covered in over 1000 and 100 studies published on the topic, respectively.[396]

10.3 Telomere length

Telomeres, first identified in the 1930s (before the establishment of the DNA double helix!)[401,402] are highly conserved regions of repetitive nucleotide sequences that protectively cap the end of all chromosomes and shorten at every cell division, thus contributing to cellular senescence. After the initial observation 30-odd years later that the ends of chromosomes cannot be replicated,[403,404] the limitation of the replicative potential of most of our cells by telomeres is nowadays a well-established fact.[405]

A large body of literature showed an association of telomere length with aging.[396,406] Especially in leukocytes, telomere shortening was associated with mortality, aging, and age-related diseases, such as cardiovascular diseases.[406–411] Moreover, gene therapy aiming at enhancing telomere length was shown to prolong the lifespan, health, and fitness in mice.[412] Increased telomere length, on the other hand, has been repeatedly associated with the risk for various types of cancer.[413] Indeed, a large meta-analysis comprising studies performed on more than a total of 1.5 million participants and covering more than 35 cancers and 48 non-neoplastic diseases, reported that long telomeres reduce the risk for some non-neoplastic diseases (including CVD), but they increase the risk for several cancers.[414] The rate of telomere shortening is suggested to be increased by a span of environmental and lifestyle factors, such as smoking, unhealthy diet, stress, lack of exercise and socio-economic status,[413,415,416] although this remains controversial.[417]

However, telomere length seems to fare poorly as a biomarker of age-induced physical decline, as measured by walking and chair-rise speed, standing balance time, and grip strength.[418] Interestingly, a study on over 1500 subjects found that the level of inflammation and not telomere length is a predictor of successful aging,[340] thus pointing to the importance of being associated with the inflammatory process for a good biomarker of biological age.

In conclusion, although telomere length is implicated in cellular aging and human diseases of premature aging, the evidence suggesting telomere length is a biomarker of aging in humans is inconclusive.[419]

10.4 Epigenetic age

The association of DNA methylation, the addition of a methyl group to a cytosine nucleotide in a cytosine-phosphate-guanosine dinucleotide (CpG), with age was originally proposed more than 50 years ago.[420] By now, this association is well established,[421–423] and exploited in different "epigenetic clocks," which linearly combine CpG methylation beta values to predict the "epigenetic age."[424,425] The first and the best known epigenetic clocks are the Horvath clock and the Hannum clock, which take into account the methylation status of 353 and 71 various CpG sites, respectively.[426,427] High epigenetic age presumably indicates poorer health. It is reported to associate with decreased physical and cognitive fitness, increased mortality risk, and many age-related and age-non related morbidities, such as Parkinson's and Alzheimer's disease, obesity, Down's syndrome, but also infectious diseases, such as HIV infection.[428–437] Indeed, a recent study on more than 9500 individuals confirmed that several different epigenetic clocks could predict the prevalence and incidence of leading causes of death and diseases.[424] Moreover, epigenetic changes appear to be reversible, which means that epigenetic clocks might be useful for validation of anti-aging interventions.[425]

However, there are several major drawbacks for using epigenetic clocks as biological age estimators: they correlate (too) highly with the chronological age,[426,427,438,439] likely only measure a phenomenon with no causal effect in the aging process,[440] and increase at a slower rate than chronological age, especially in the senior population.[441]

10.5 Glycan age

The association of IgG glycome composition with chronological age has been amply described in Section 8. However, this association is weaker than the one of DNA methylation with chronological age. IgG glycome alone allows for a prediction of chronological age with an estimated error of 9.7 years.[36] The difference between the estimate and the actual chronological age is suggested to relate to biological age - after the correction for chronological age, IgG glycome associates with many biochemical and physiological traits related to inflammation and poor metabolic health, including: serum glucose and insulin levels, hemoglobin A1c, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, waist circumference, body-mass index, fibrinogen, d-dimer, uric acid, creatinine, alanine aminotransferase, aspartate aminotransferase, C reactive protein, waist-to-height ratio, etc.[31,36,331,442] Additionally, a study on 27 populations sampled worldwide found that Fc IgG galactosylation, especially monogalactosylation, positively correlated with the expected lifespan.[24]

The capacity of IgG glycome for immune response modulation and its plasticity in response to various environmental stimuli makes it an obvious candidate for a marker of biological age. Its unique feature of combining genetic background with consequences of environmental effects and the immunological aspect of aging makes the IgG N-glycome a potential "Holy Grail" for biological aging.[250,443] This is supported by the fact that many autoimmune, inflammatory, and other diseases and aging share a common IgG glycosylation pattern: a decreased level of terminally galactosylated and terminally sialylated glycoforms, and an increased level of glycoforms with bisecting GlcNAc,[32] thus positioning IgG glycoprofile as a read-out of a general state of health, i.e. a biomarker of general immune activation.[37]

Excitingly for the prospect of improving one's biological age, the IgG glycomic profile seems to be responsive to certain lifestyle changes. A longitudinal study on exercise-induced weight loss found that energy deprivation achieved by dietary restriction in combination with prolonged intense exercise resulted in a general suppression of both innate and adaptive immunity, accompanied by a pro-inflammatory IgG glycoprofile.[41] In contrast, various other interventions described in longitudinal studies, such as

repeated sprint training, the introduction of dietary supplements, estrogen supplementation, and extensive weight loss after bariatric surgery were confirmed to change the subjects' IgG glycosylation pattern from pro- towards anti-inflammatory, i.e. improve the "glycan age."[38–40,43,346]

10.6 Composite biomarkers of biological age

Given the daunting complexity of the aging and age-related pathological processes, as well as the system specificity within the human organism, it seems highly unlikely that any single read-out will ever be able to represent the whole span of events involved in biological aging. Research is now, therefore, very much focused towards the integration of multiple -omics data for the identification of composite panels of biological age biomarkers, which will integrate diverse measures of selected biological properties.[444] In parallel, diverse interventions aimed at prolonging the lifespan and improving quality of life are proposed and tested.

11 Future of IgG glycosylation

What does the future of IgG glycosylation hold? (How) will it be harnessed and exploited for our benefit? In this section we offer some of the still open scientific questions and areas worth of research, some of them already under way.

11.1 Methodology

An immense amount of effort has already been invested in the advancement of analytical methods aimed at IgG glycome analysis, particularly in the last decade. This includes decreasing the limit of detection, better glycoform separation, more precise structural analysis, high throughput, and analysis of non-standard biological samples, such as cerebrospinal and synovial fluid, method validation, standardization, comparison, and automatization.[34,81,170,183,186,188,199,202,203,225,359,445–457] Since scientific discoveries in this field are still highly dependent on the performance of analytical tools, method improvement is always welcome; particularly if it permits IgG glycan analysis to become rapid, affordable enough for biomarker discovery or routine clinical use and allows the analysis to be performed after a simpler and less invasive sample collection, such as dried blood spots and saliva.[82,458] To support the acquisition and automated analysis of such a vast quantity of data, advanced bioinformatics tools are already being built,[459–461] and are expected to advance in order to enable the seamless integration of the IgG glycomics data with the other -omics layers of the collected metadata.

11.2 Animal models

Mouse is routinely used as a model organism for research of human biological processes. It is particularly suitable for intervention studies aimed at establishing the causality that are not feasible on human subjects due to ethical constraints. IgG glycosylation is no exception. Despite some dissimilarities (different sequence and functionality of IgG subclasses and FcγRs, the presence of galactose-α-1,3-galactose epitope and *N*-glycolylneuraminic acid instead of *N*-acetylneuraminic acid), mice share many IgG-related features with humans, making them a good experimental model for examining IgG glycosylation.[462,463] Research on mouse IgG glycosylation has intensified in the last couple of years,[15,38,42,259,279,462–466] but it needs to be explored in further detail, allowing us to become aware of the exact limitations of particular models. Some of the outstanding questions in this regard are: Does mouse IgG glycosylation follow the same dynamics patterns as human IgG in aging and various diseases? Which strains are optimal for different types and topics of study?[463,466] Prerequisites for answering these questions are method optimization for mouse IgG glycosylation analysis[467,468] and close examination of particularities of mouse IgG glycosylation – such as the existence of polymorphic variants next to the conserved N-glycosylation site in IgG1 that seem to influence the IgG glycoprofile.[208]

Compared to mice, very little is known of IgG glycosylation in non-human primates.[469] Since they are often used in preclinical studies during vaccine and mAbs development, this important aspect of their immune response should not be neglected and more research in this area is required.

11.3 Regulation and functionality

The necessity to solve the open question of IgG glycome composition regulation has already been extensively addressed in Section 6. Answering it could to enable us to both regulate IgG glycosylation to our advantage, and identify and explain the molecular pathology of diseases in which IgG glycans play a functional role.

11.4 Personalized medicine - Disease prediction and diagnosis, patient stratification, monitoring of disease progression and response to therapy

As already explained in Section 10, the IgG glycosylation pattern can be associated with unhealthy aging, an increased risk of disease development, or pre-symptomatic disease development. The role of IgG glycosylation analysis in this set-up would be to serve as a

signal for the necessity of a lifestyle change or other type of intervention, ideally resulting in healthier aging, disease prevention, and early detection.[470,471]

In pathological settings, such as RA, Crohn's disease, tuberculosis, and several types of cancer, in which IgG glycome composition has been shown to associate not only with the diseased state in general but also with symptom severity, disease progression, as well as with the response to therapy, IgG glycome evaluation is suggested as a useful add-on tool for monitoring disease progression and therapy response.[215,219,220,246,348,350,358,472–475]

Additionally, looking into IgG glycosylation in concert with other clinical data might also shed light on underlying pathological mechanisms of the disease and therapy mode of action on a molecular level.

Perhaps the most exciting prospect of personalized medicine proposes that patient stratification should allow for better differential diagnosis and, consequently, therapy selection. Indeed, a higher level of IgG sialylation predicted therapy response in Kawasaki disease,[476] while a higher level of IgG galactosylation predicted response to anti-tumor necrosis factor (TNF) therapy in RA and Crohn's disease.[472] Likewise, high Fc galactosylation is associated with response to methotrexate therapy in RA.[477] In another study, however, serum N-galactosylation (mostly reflecting IgG galactosylation) failed to predict the response of RA patients to therapy with methotrexate and TNF blockade.[478] IgG core fucosylation has been proposed as a potential marker for improved patient stratification, aimed at the identification of patients at risk for a serious disease phenotype in alloimmune conditions FNAIT and HDFN.[363–366] These studies offer a glimpse of hope that chronic patients might benefit from the developments in the IgG glycoscience, but there is still a long road ahead before any of these basic discoveries are validated and potentially translated to the clinic. To our knowledge, the only currently available glycosylation-based test is aimed at the stratification of patients according to a range of hepatic categories (ranging from liver inflammation, through early and late fibrosis to early cirrhosis and prediction of hepatocellular carcinoma development) based on total serum N-glycans,[479–482] some of which stem mainly from IgG.[45]

In summary, although there is clearly a potential in using IgG glycosylation analysis to achieve each of the multiple goals of personalized medicine, the data currently at hand is partly inconclusive. Therefore, this concept remains to be thoroughly investigated and validated before a possible translation to the clinic.

11.5 Glycoengineering of therapeutic monoclonal antibodies

Tailoring the glycosylation of mAbs to achieve the desired functions is probably the most prominent application of IgG glycosylation, well-known even outside the glycoscientific community. This is also reflected in the fact that glycosylation is one of Critical Quality Attributes for the produced mAbs.

In cases where the mAb mode of action relies on one of the mechanisms modulated by IgG glycosylation, such as ADCC, ADCP, and CDC, minute changes in Fc glycan composition can have immense consequences on clinical efficacy.[483] The most prominent example is the enhanced cytotoxicity of anti-tumor mAbs achieved through elevated binding to FcγRIIIA, which arises from a lack of core fucose. One of the examples of such successful glycoengineering is the improvement of effector functions of rituximab, an anti-CD20 mAb approved for the treatment of B-cell malignancies, by increasing the abundance of afucosylated glycoforms.[484–488] Indeed, today mAb glycoengineering is mostly used for the production of marketed afucosylated antibody glycoforms. Since microbial expression systems are incapable of appropriate N-glycosylation, mammalian - and in particular Chinese hamster ovary (CHO) - cells are used for the production of a vast majority of therapeutic mAbs.[145,489] Therefore, the mAb glycoengineering often focuses on the manipulation of the host synthetic pathways to achieve the desired, nearly homogenous glycoform.[142] A prominent example of such approach is knocking down or knocking out the FUT8 gene,[490,491] although other approaches are also in use, including *in vitro* chemo-enzymatic remodeling.[489] The vast expanse of this topic is out of the scope of this chapter and has been extensively reviewed elsewhere.[21,489,492–494]

11.6 Tailoring IVIg glycosylation

The finding from animal autoimmune models that the anti-inflammatory therapeutic effect of IVIg administered at high (g/kg) doses might be predominantly mediated via its sialylated Fc glycans[178] implies that the sialylated IVIg fraction might prove more efficient in the alleviation of autoimmune conditions. However, contrary to expectations, SNA lectin fractionation of IVIg did not prove successful in a model for immune thrombocytopenia, perhaps due to the fact that only Fab and not Fc sialylated IgG was enriched by this protocol.[301] A different approach, aiming at the hypersialylation of IVIg, while avoiding other unwanted alterations, proved more successful. Tetra-Fc-sialylated enzymatically modified IVIg showed 10-fold higher anti-inflammatory activity across four different animal models: immune thrombocytopenia purpura, inflammatory arthritis induced by serum transfer or anti-collagen antibodies, and epidermolysis bullosa acquisita.[296] The clinical evaluation of this drug candidate for the treatment of immune thrombocytopenia purpura is under way.[495] Alternative chemoenzymatic platforms for the production of hypersialylated IVIg are being tested as well.[496]

Besides mAbs and IVIg, many IgG-based biopharmaceuticals would probably profit from glycosylation optimization, including Fc fusion proteins and polyclonal IgG preparations for prophylaxis and passive immunotherapy (HIV, rabies, anti-rhesus).[494,497,498]

11.7 Therapeutic administration of enzymes modulating endogenous IgG glycosylation

Perhaps currently still the most far-fetched of all mentioned here, this concept proposes that in vivo administration of enzymes for IgG glycan modification or targeted removal could be used in therapeutic purposes. The idea is based on disease abrogation following Fc deglycosylation by Endo S in a variety of preclinical autoimmune models, including RA, SLE, skin-blistering diseases, and immune thrombocytopenic purpura (ITP).[19,143,499,500]

Likewise, following the discovery that the sialylated IVIg fraction was responsible for the anti-inflammatory activity of IVIg administered at high (g/kg) doses,[178] it was proposed that elevating the fraction of sialylated bulk serum IgG antibodies to a certain critical level might be sufficient to attenuate inflammatory autoimmune conditions. Indeed, in vivo engineered sialylation of pathogenic autoantibodies was found effective at attenuating autoimmune inflammation similarly to IVIg administered at a high doses.[501]

Due to possible unforeseen consequences, however, in vivo administration of enzymes aimed at IgG glycan modification should be examined extremely carefully before possible application in human patients.

11.8 Optimization of vaccination protocols aiming at elicitation of targeted IgG glycoforms

Studies showing that antigen-specific IgG elicited in the context of natural immunization (HIV, hepatitis B and C virus, dengue virus, cytomegalovirus, mumps, SARS-CoV-2), inflammatory diseases (granulomatosis with polyangiitis), allo- and autoimmune pathology, (FNAIT, HDFN and RA, SLE, anti-neutrophil cytoplasmic antibody-associated vasculitis, autoimmune hemolytic anemia, respectively) immunization by vaccination (tetanus, influenza, mumps, measles, parvovirus, HIV, pneumococcal and meningococcal vaccine), allergen-specific immunotherapy and cancer (ovarian cancer) can have a different glycosylation pattern from the bulk IgG,[222–225,236,354,358,363–367,382,384,388,464,502–512] point to the precise regulation of IgG glycosylation. Together with the confirmed capacity of differential glycosylation to modulate the downstream IgG effector functions, this notion implies that the elicitation of particular glycoforms might be a beneficial and therefore desired effect of vaccination.

This was confirmed by a clinical study in which healthy volunteers received a trivalent inactivated influenza vaccine. The abundance of Fc-sialylated anti-hemagglutinin IgG predicted the vaccine efficacy, as measured by hemagglutination inhibition titer.[513] A similar approach might prove useful for the optimization of allergen-specific immunotherapeutic preparations for the treatment of allergic diseases.[236,464]

Regarding the possible use of targeted IgG glycosylation for vaccination purposes, several questions remain, coming back to IgG glycosylation regulation: If a targeted IgG glycopattern can be established, how long post-vaccination will it persist? Will the vaccination-induced long-living plasma cells keep the same IgG glycoprofile or will it be modified by a changed immune context at a later point in time? Will the antigen-specific memory B cells recruited following the secondary exposure secrete IgG with the same glycosylation pattern that was established during the initial exposure? Time and research will tell.

11.9 The future

Taking into consideration the marked shift in the field of IgG glycosylation research towards biomarker discovery and mechanistic involvement of IgG in immune processes, which has happened in the last several years,[13] we can be hopeful that at least some of the open questions will be addressed in the near future. This would allow the knowledge on IgG glycosylation to be of use in a range of biomedical applications, from immunotherapy and vaccination to healthy aging and disease prevention. However, since IgG glycosylation is involved in a plethora of different pathways (the details of them often remaining unexplored for most of the conditions), we need to be extremely mindful of potential unwanted consequences. Affecting the IgG glycosylation in a narrow niche (e.g. by optimizing the IgG glycosylation pattern of antigen-specific IgG elicited by vaccination) might be relatively easy to achieve and follow up. By contrast, affecting the glycopattern of the whole serum IgG might bear extensive and unforeseeable consequences on the whole immune ecosystem of an individual.

12 Conclusions

At first sight, compared to some other proteins, IgG glycosylation should be rather simple and predictable. There is but one conserved N-glycosylation site in the Fc region of each of IgG heavy chains, and only a minority of the Fab regions are glycosylated due to the acquisition of new glycosylation sites during somatic hypermutation. The number of antennas never seems to exceed two in both of these regions, thus further limiting structural diversity.

However, having considered all written above, it is now clear IgG glycosylation is all but "simple." This is the case when it comes to IgG glycoforms structural heterogeneity, but even more so when it comes to functional implications of differential IgG glycosylation. Many of the questions mentioned in the introduction remain open, but some ideas are taking shape and have become accepted, albeit to different degrees:

The IgG glycoform pool is very large within each individual. Polypeptide sequence differences aside, this pool likely consists of more than a thousand different IgG glycovariants, the number reflecting 30-odd distinct N-glycan structures, asymmetrical N-glycan

pairing in the Fc region, non-conserved glycosylation sites in the Fab region, asymmetrical Fab glycosylation and Fc O-glycosylation of IgG3.

There is a large variability in IgG glycome composition within human populations, with galactosylation being the most variable trait.

Minute differences in IgG glycan composition (differential glycosylation) influence the interactions of both Fab and Fc region with its natural ligands, thus affecting their downstream functions. In general, agalactosylated, asialylated, and bisected IgG molecules are considered "pro-inflammatory." By contrast, galactosylated and sialylated IgG molecules are often described as "anti-inflammatory." Afucosylated IgG has a much higher capacity to induce ADCC via FcγRIIIA binding.

Regulation of IgG glycosylation is very complex and still vastly underexplored. It is known, however, that it is under significant influence of both genetic make-up and environmental factors, among others. The question remains how much of any given IgG glycopattern is just a consequence of the general inflammatory status and how much is purposefully directed for the modulation of effector functions.

The inflammaging theory proposes that the gradual accumulation of pro-inflammatory IgG glycoforms, in turn, serves as one of the effector mechanisms of aging, enhancing inflammation in a vicious self-fuelling loop.

Many inflammatory, autoimmune and neoplastic diseases share the glycosylation profile of bulk (total serum) IgG characteristic for aging (decreased level of galactosylated and sialylated structures, increased level of structures with bisecting GlcNAc), likely due to common underlying inflammatory component of disease pathology. In this case, this specific IgG glycopattern could reflect a predisposition towards disease development, or even be involved as an effector of the inflammation. On the other hand, it might reflect environmental exposure, such as antigen load or unhealthy lifestyle. This makes the IgG glycoprofile read-out one of biomarkers of biological age, i.e. a general state of health.

In the case of a distinct glycopattern of antigen-specific compared to bulk IgG, it seems more likely that IgG glycans are directly involved in disease pathogenesis and progression through specialized effects highly dependent on the disease/condition.

First intervention studies influencing the IgG glycome offer a hope that in the future, we might have a chance to prevent or revert the age- and/or disease-associated phenotype by influencing our IgG glycosylation pattern.

IgG glycosylation will hopefully play a more prominent role in the area of personalized medicine, aiming at improved disease prediction and diagnosis, patient stratification, monitoring of disease progression, and response to therapy.

Tailoring IgG glycan composition to better suit the intended function is a means to optimize all IgG-based therapeutics applied to humans, as is currently the case for monoclonal antibodies.

Whether the more far-fetched prospects, such as optimization of vaccination protocols for targeted glycosylation of anti-pathogen IgG and enzymatic in vivo modulation of endogenous pathological IgG glycosylation, will prove successful and safe enough to warrant the use in humans remains to be seen.

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