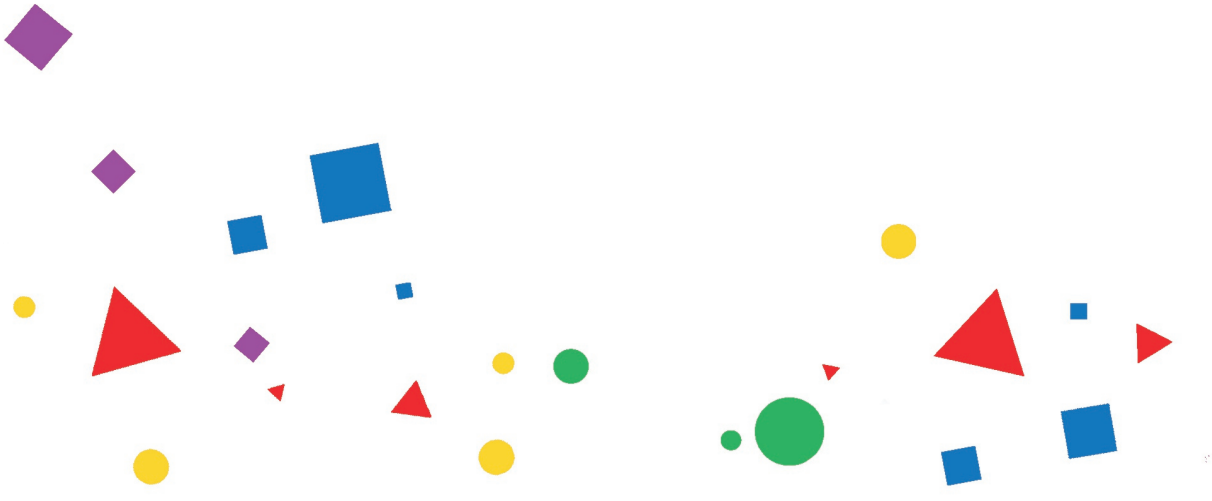
Glycosylation analysis of immune-related molecules

Borosak, I.



Chapter 1

General introduction and scope of the thesis

1. INTRODUCTION

Herein, I discuss the importance of glycosylation of key components/proteins of the immune system and its perturbations in autoimmune and infectious diseases. Moreover, I describe how state-of-the-art mass spectrometry (MS) technologies can be used to identify the glycan signatures of different autoimmune and infectious diseases.

1.1 BACKGROUND

Glycosylation is a common feature of the surfaces of all living organisms and most secreted proteins. Together with nucleic acids, proteins and lipids, glycans are one of the four fundamental classes of molecules that they are essential for life. In the immune system, glycans participate in nearly all immunological processes, including host-pathogen interactions, immunological recognition and activation, and differentiation between self and non-self-antigens. Proper glycosylation is crucial for maintaining homeostasis, while changes in glycosylation patterns can broadly impact fundamental immunological processes. Alterations in the glycan repertoire on various immunological components may be triggered by environmental and genetic factors. Aberrant glycosylation of immune components is associated with many inflammatory, autoimmune and oncological diseases. Though regulation of glycan changes is not well understood, current methodological advances have enabled association of glycosylation changes with the onset, progression and outcome of many pathologies. Especially, dysregulation of N-glycosylation is implicated in many immune-related disorders. Therefore, understanding the role of N-glycosylation in immunity and inflammation is important for developing new diagnostic tools, therapies and treatments for these conditions.

1.2 PROTEIN GLYCOSYLATION

Protein glycosylation is the enzyme-catalyzed process of the addition of a carbohydrate chain, or glycan, to a protein backbone. The process takes place in the endoplasmic reticulum and Golgi apparatus, where secreted and membrane proteins are being glycosylated, including key components of the immune system, such as antibodies, cytokines and chemokines as well as their receptors.(1) Protein glycosylation comes in two main classification types: N- and O-glycosylation. The glycan class is defined based on the side-chain atoms of amino acid to which glycans are linked. In N-glycosylation, glycans are attached to the nitrogen atom of an asparagine (Asn, N) side chain by a β-1N linkage, whereas in O-glycosylation glycans are attached to the oxygen atom in the side chain of serine and threonine residues.(2) Comprising often a significant proportion of the total glycoprotein size, glycans profoundly modulate physicochemical properties, structure, and function of the conjugated proteins. In addition, glycans mediate a wide range of biological roles, as well as intrinsic and extrinsic recognition.(2) Recent technological advances have provided new insights into the structure of the glycans and glycoconjugates and revealed their diversity.(3) Furthermore, an increasing number of large cohort studies have profiled and compared glycosylation in physiological and pathophysiological states, enabling glycan-based clinical marker discovery.(4)

1.2.1 Protein N-glycosylation

Whether a protein is glycosylated or not depends mainly on the presence of the consensus sequence Asn-X-Ser/Thr (X≠Pro) and occasionally Asn-X-Cys. Around 70% of proteins are estimated to contain the consensus N-glycosylation sequence. However, not all sequons are N-glycosylated and the average N-glycan site occupancy is estimated at 65%.(5)

N-glycan processing begins with the transfer of a 14-sugar oligosaccharide to the sequon. Subsequently, the attached precursor glycan is trimmed by series of glucosidases and mannosidases in the ER. It undergoes further maturation in the Golgi by adding monosaccharides, such as N-acetylglucosamine, galactose, sialic acid and fucose.(2) Based on the final structure, N-glycans can be classified into three main classes: oligomannose, hybrid and complex. The three different classes share a common pentasaccharide core consisting of two GlcNAcs and three mannose residues. Oligomannose N-glycans exist with the core structure and the addition of up to six mannose residues. These glycans undergo only incomplete processing and escape further modifications. In contrast, complex type N-glycans contain one to four additional GlcNAc residues, so-called antennae, which are often elongated with galactose and sialic acid. And as its name implies, the hybrid type is a combination of both and has one complex arm bearing at least a GlcNAc, and one arm terminated with at least one additional mannose.

Unlike genome, transcriptome or proteome biosynthesis, glycan biosynthesis is a non-templated process and thus, relies on the combinatorial activity of subsets of glycosyltransferases and glycosidases regulated by many cellular factors. These include cell type, cell signals, the availability of nucleotide sugars, and the accessibility of the N-glycosylation site. Moreover, N-glycan compositions depend on the physiological state of the cell and variations occur with almost every disease. Thus, the final N-glycan compositions on a given protein are very diverse and vary from tissue to tissue, cell to cell, protein to protein, and even site to site reflecting the overall cellular status in health and disease. To describe the variation in protein glycosylation, terms such as macro-heterogeneity and micro-heterogeneity have been introduced. Macro-heterogeneity describes the diversity in glycan occupancy, while micro-heterogeneity refers to the variations of glycan structure at a specific site. Importantly, both forms of heterogeneity strongly influence many of glycoprotein functional aspects and reflect a given physiological and pathophysiological state.

1.2.2 N-Glycosylation in immunity and inflammation

Glycans are present on key immune molecules and play a role in regulating their interaction, signal transduction and effector functions. They control molecular interactions and thus convey important functions in the innate and adaptive immune response through several basic mechanisms. The glycan recognition system, for example, promotes biological activity via binding of distinctive glycan moieties on the surface of soluble proteins or immune cells to glycan-binding proteins (GBPs), such as lectins. The intimate contact of GBPs and specific

carbohydrate determinants on respective ligands is essential for maintaining homeostasis and regulating immune and inflammatory responses, including immune cell trafficking, signalling and maintenance of self-tolerance.(6, 7)

In the adaptive immune system, glycans present on secreted or cell surface molecules can modulate signal transduction, protein-protein and cell-cell interactions necessary for humoral and cellular immunity. For instance, glycans found on T-cell (TCR) and B-cell (BCR) receptors, along with their cognate lectins are involved in the molecular mechanism that controls the threshold of TCR and BCR activation. Changes in glycan composition modify these molecular interactions, thereby influencing immunological signaling with a profound effect on the immune system.(8, 9) In addition to their presence on the cell surface molecules, glycans also regulate the immunological functions of secreted proteins such as immunoglobulins (Igs), or antibodies. Igs are essential components of humoral immunity that recognize, neutralize and mark pathogens, such as bacteria, fungi or viruses, for elimination. The glycans on Igs can affect their structure, half-life, antigen- and receptor-binding properties, ultimately influencing their effector and biological functions.(10, 11) Alterations of IgG glycosylation affect binding to Fc gamma receptors (FcγRs) and complement component 1q (C1q), and thus downstream effector signaling, such as ADCC and complement activation.(12, 13) Changes in Ig glycosylation have been linked to various diseases, including autoimmune disorders and cancer.(14)

1.3 IMMUNOGLOBULINS

Immunoglobulins are glycoproteins produced by effector B-cells (plasma cells) and constitute about 20% of the protein content of plasma. As indicated in **Figure 1A**, the immune system produces five classes or isotypes of antibodies, namely IgG, IgA, IgD, IgE, and IgM, each of which contains a distinct heavy chain – gamma, alpha, delta, epsilon, and mu, respectively.(15) In addition, there are two types of light chains - kappa and lambda, with kappa being the more common of the two in humans. The two arms of the Y-shape form the fragment antigen-binding (Fab) region, which recognizes and binds to specific epitopes on target molecules. In contrast, the fragment crystallizable (Fc) region of the molecule, located at the base of the Y, mediates different effector functions through the interaction with Fc-engaging molecules.

IgG, which is the most abundant antibody in the blood stream, is produced in response to both acute and chronic infections. It can neutralize pathogens, activate complement, and facilitate opsonization and phagocytosis by immune cells.(16). IgM is the first antibody produced in response to infectious agents and antigens and is present in a monomeric form on the surface of naïve B cells as a B-cell receptor. Secreted IgM is a pentamer and has ten antigen-binding sites, allowing it to bind multiple antigens simultaneously and initiate the classical pathway of the complement activation. IgA is primarily found in mucosal secretions such as saliva, intestinal fluid or breast milk, where it plays a critical role in preventing pathogen entry into the body. IgD is found on the surface of naïve B cells and is involved in

B cell activation and differentiation. IgE is primarily involved in allergic responses.(15). Each Ig isotype can elucidate a wide range of partially unique effector mechanisms, resulting in responses against pathogens and the maintenance of immune homeostasis.

Regardless of the isotype, all immunoglobulins are glycosylated in the constant region and glycosylation is of great importance for the appropriate function of Igs. The number and position of glycans vary between different Ig classes. While IgA, IgM, IgD and IgE have several conserved N-glycosylation sites in the Fc region, accounting for approximately 12-14% of their molecular weight, IgG is characterized by only one N-glycosylation site.(17) In addition to N-linked sugar moieties, IgA1 and IgG3 also contain O-glycans in their hinge region.

Ig effector functions are dynamically regulated by various modification of the antibody Fc region, with glycosylation being one of the factors that exerts a profound impact on the Igs' biological activity. Furthermore, glycosylation patterns on Ig molecules can vary depending on cell type, tissue location, and disease state. Such variation in glycosylation can modulate antibody effector functions, such as complement activation, antibody-depended cellular cytotoxicity, and clearance from circulation.(18) There is no conserved N-glycosylation site in the Fab region. However, N-glycosylation sites can be generated de novo by somatic hypermutation during the antigen-specific immune responses.(19). All in all, Ig glycosylation is a highly regulated process with a strong impact on Ig effector functions.

1.3.1 IgG structure and glycosylation

Glycosylation is thus of great importance for the proper function of all Igs. The IgG class of immunoglobulins is divided into four subtypes (IgG1, IgG2, IgG3, IgG4), which can be further subdivided into their polymorphic variants, called allotypes. (20). The schematic representation of IgG subclasses and their glycosylation is shown in **Figure 1B.** IgG subclasses and allotypes differ in the amino acid sequence of the heavy chain of the Fc region, a region that interacts with Fc-engaging molecules, such as FcγRs or C-type lectins. As a result of these variations, each IgG subtype poses unique structural and functional characteristics, as well as, a unique profile with respect to triggering effector mechanisms, including antibody-depended cytotoxicity (ADCC), antibody-depended phagocytosis (ADCP), and complement-depended cytotoxicity (CDC). For example, IgG1 and IgG3 have a longer hinge region that allows for increased flexibility and range motion, which is important for their ability to facilitate effector functions. IgG2 and IgG4 exhibit shorter hinge regions, which limits their ability to engage effector cells. Instead, these subtypes play a more prominent role in neutralizing toxins and antigens. IgG subclasses have different patterns of expression in response to different types of antigens. For example, IgG1 and IgG3 are more commonly produced in response to protein antigens associated with viral infections, IgG2 is produced in response to polysaccharide antigens induced by bacterial infections, and IgG4 is formed in chronic exposure to allergens.(20, 21)

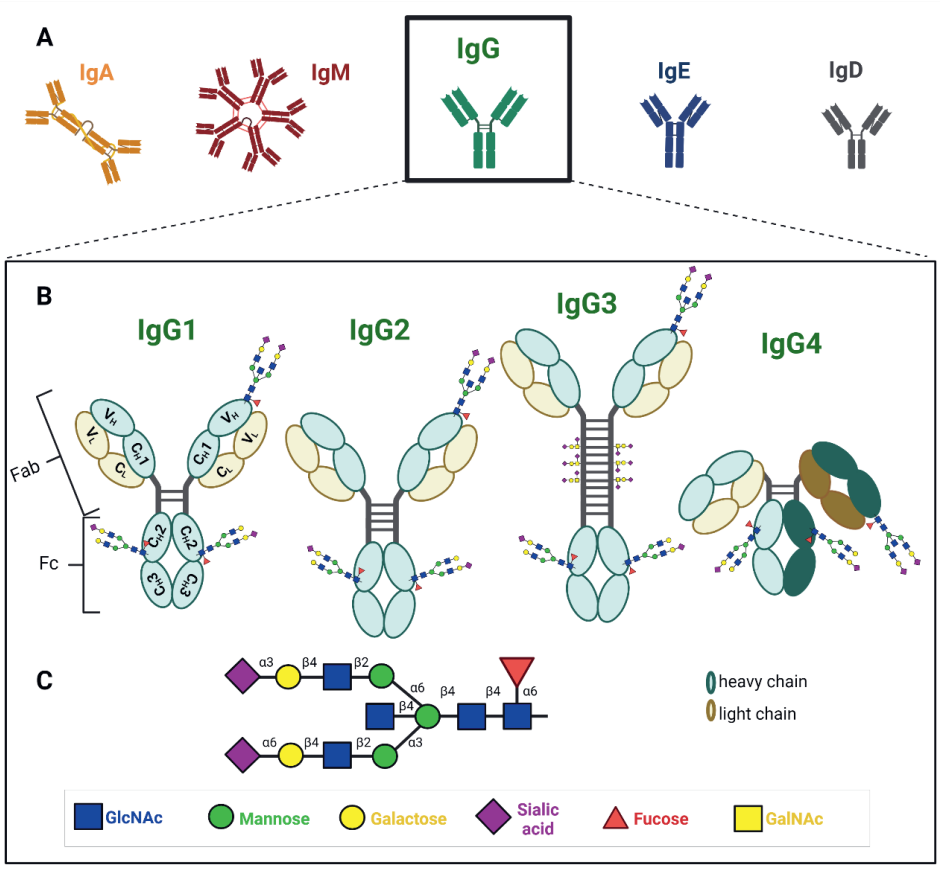


Figure 1. *Immunoglobulins and their glycosylation. (**A**) Immunoglobulin (Ig) isotypes. The basic functional unit of each antibody consists of four polypeptide chains, typically two identical heavy (H) chains and two identical light (L) chains. (**B**) A structural representation of the Immunoglobulin G (IgG) subclasses and their glycosylation. Regardless of the isotype, subtype and allotype, the heavy chain consists of three constant (C) domains (C_H1, C_H2, C_H3), and a variable (V) domain (V_H). The light chain contains two domains, one constant (C_L) and one variable (V_L). Fab and Fc are connected via the hinge region. IgG3 has a longer hinge region bearing O-glycans. The bispecific nature of IgG4 is indicated by slightly darker colors. (**C**) Symbolic representation of the most elaborate, complex type N-glycan found on IgG. Created with Biorender.com*

IgG contains an N-liked glycosylation site at the conserved asparagine 297 residue in each of the C_H2 domains of the Fc region of the molecule. Around 30 different N-glycans decorate this single N-glycosylation site.(22) Unlike other plasma proteins which are lacking core-fucosylated glycans, plasma IgG of healthy individuals contain on average over 90% core-fucosylated glycans, that is fucose attached to the innermost GlcNAc of the N-glycan core. Moreover, these fucosylated and complex-type glycans carry intermediate levels of galactose (30-65% per antenna, low levels of bisecting GlcNAc (10-20%) and low levels of sialic acid (10-15% per antenna).(22) In addition to all IgG being glycosylated at the Fc region, 10-15% of IgG undergo glycosylation in the Fab portion.(19) The absence of a conserved N-glycosylation site complicates and therefore limits research on glycans linked

to the IgG Fab region. Nevertheless, it is established that these N-glycans are, like Fc glycans, mainly complex-type biantennary structures. However, compared to IgG Fc glycans, Fab glycans are characterized by the presence of fully processed sugar moieties, rich in sialic acid (~80%), bisecting GlcNAc (~65%) and galactose (~97%) residues in addition to exhibiting a lower fraction of core fucose (~70%).(23, 24) In addition to the impact on antigen binding, Fab glycosylation also affects the stability and half-life of IgG.(10)

Within the individual, the glycosylation profile is rather stable over time under homeostatic conditions, but it can change significantly with altered physiological or pathological states. Variations in the glycosylation profile of serum IgG have been correlated with age, inflammatory conditions, infectious diseases and cancers.(14)

1.3.2 The impact of glycosylation on the function of IgG

IgG exert their biological activity through the induction of effector mechanisms, such as complement activation and FcγR-depended effector functions (**Figure 2**). IgG effector functions and stability are heavily influenced by the carbohydrate moiety attached to the Fc domain. Based on the composition of this moiety, IgG are capable of exerting both pro- and anti-inflammatory effector functions. The presence of N-glycans changes the conformation of the Fc region and supports its open conformation, allowing interaction with the Fc-engaging molecules.(25) Thus, the complete removal of Fc glycans changes the conformation from open to closed and diminishes the functional binding to Fc receptors and C1q.(10, 26) Alterations in the composition, size and charge of the Fc N-glycan also lead to conformational changes in the Fc region with a profound impact on binding properties to interaction partners. Thereby, Fc N-glycosylation modulates IgG's ability to activate the classical complement pathway and FcγR-expressing effector cells, impacting such effector functions as complement-dependent cytotoxicity (CDC) and antibody-depended cellular cytotoxicity (ADCC).(18)

In recent decades, studies have demonstrated that IgG core fucosylation, which refers to the addition of a fucose residue to the innermost GlcNAc by an α1,6-fucosyltranserase (FUT8), has a profound impact on IgG depended effector functions, especially ADCC activity mediated by natural killer (NK) cells.(13, 27) Antibodies deficient in core fucose bind with 10-100 fold higher affinity to FcγRIIIa and FcγRIIIb with respect to its fully fucosylated counterparts.(27, 28) This in turn, results in 2-40 fold increase in ADCC. Mechanistically, the higher affinity of afucosylated IgG is explained by a more favourable carbohydrate-carbohydrate interaction between the N-glycans of IgG-Fc and FcγRIIIa.(29)

IgG sialylation was shown to have anti-inflammatory effects. A classic example of anti-inflammatory activity is the discovery that infusion of high doses of intravenous immunoglobulin G suppresses a wide variety of inflammatory and autoimmune diseases.(30, 31) However, when terminal sialic acids are removed from IgG, they become more pro-inflammatory and may contribute to the development of inflammation. The precise mechanisms by which sialylated IgG interact with immune cells and modulates the balance between pro- and anti-inflammatory activity are not yet fully understood. More

functional studies are needed to resolve some of the apparent discrepancies.(32) Fc-sialylated glycovariants have been found to decrease affinity for activating FcγRs and C1q, resulting in a reduced capacity to trigger pro-inflammatory IgG effector functions, ADCC and CDC, respectively.(33, 34) Indeed, it was suggested that the sialic acid causes conformational changes that impede the binding of FcγRIIIa and FcγRIIIb. Moreover, it was suggested that IgG sialylation exert its anti-inflammatory activity through the induction of IL-33 production, which in turns upregulates FcγRIIb expression on effector cells.

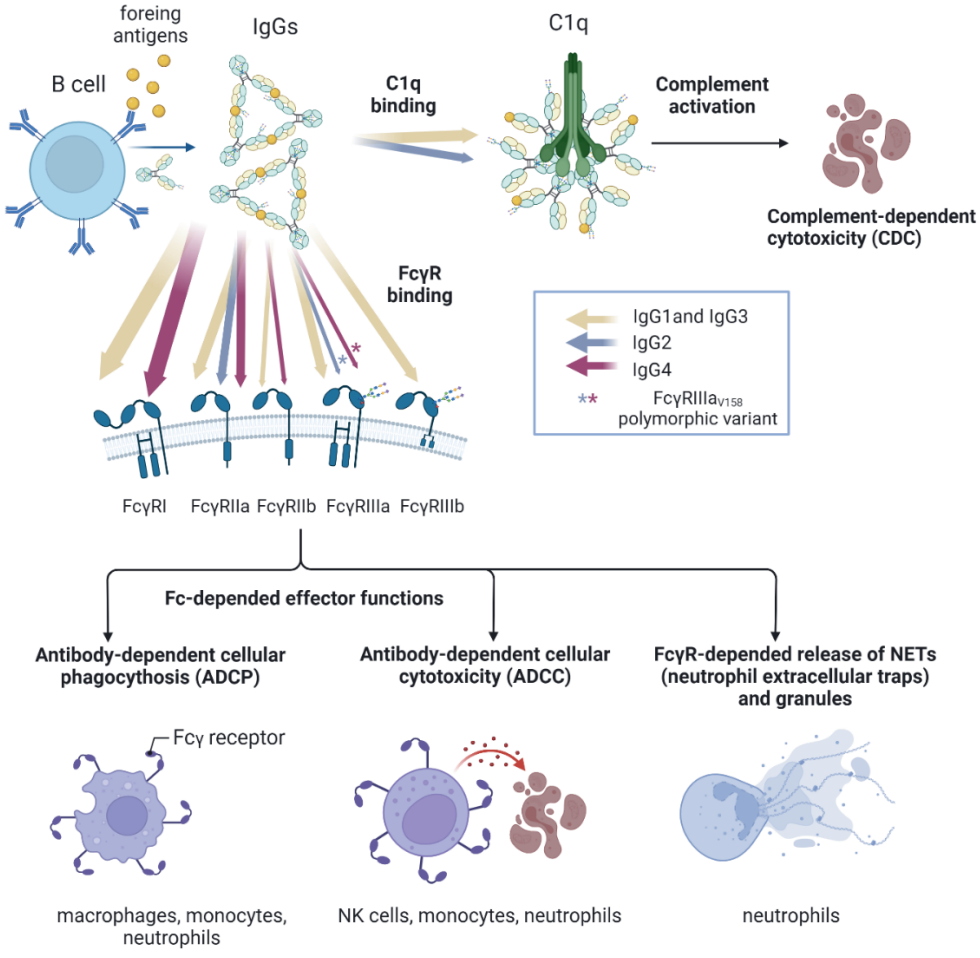


Figure 2. *The diverse repertoire of immunoglobulin G effector functions. Interaction of IgG-Fc with C1q activates the classical complement pathway, while its interaction with FcγRs elicits antibody-dependent cellular cytotoxicity (ADCC) and antibody-depended cellular phagocytosis (ADCP). Human IgG subclasses have different affinity for FcγRs which is indicated by the colour of the arrows (subclass) and their thickness (thicker denotes higher affinity). IgG1 and IgG3 are highly efficient in inducing effector mechanisms, whereas IgG2 and IgG4 are much less efficient and only do so in specific cases.(20, 35) Created with Biorender.com*

However, one study showed little impact of Fc-sialylation on ADCC, and another even demonstrated increased C1q binding. Some models support a role of sialic acid in promoting binding to human type II lectin receptors (DC-SIGN, CD23).(13, 36) However, there are conflicting reports and whether there is a direct interaction with DC-SIGN is under debate.(37)

The addition of a β1,4-linked GlcNAc to the core β-mannose residue, referred to as bisection, results in a conformational alteration of the N-glycan structure.(38) However, this modification does not seem to have any significant functional consequences since it has a very limited impact on FcγR or C1q binding.(13, 39) Despite the uncertain significance of bisection to antibody effector functions, it appears to have an indirect effect on IgG effector functions by suppressing subsequent fucosylation during N-glycan synthesis.(40)

The role of IgG Fc-galactosylation in modulating Fc-mediated effector functions is complex and multifaceted. IgG lacking terminal galactose residues has been shown to promote pro-inflammatory activity via the lectin complement pathway upon binding to mannose-binding lectin.(41) However, later studies have challenged this observation.(42) More recent data have shown that elevated galactosylation may act in a pro-inflammatory fashion by increasing binding to FcγRIII and consequently NK cell-mediated ADCC, especially in synergy with afucosylation.(13) Likewise, by increasing the affinity of IgG for the C1q complement component, galactosylation enhances CDC, at least in vitro.(13, 43) Mechanistically, galactosylation enhances IgG1 hexamerization potential, and thus, subsequent downstream activation.(44)

Antibody glycosylation is largely determined by the abundance and activity of glycosyltransferases and glycosidases which, in turn, depends on the availability of sugar donors, transcription factors, and cytokines, such as the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα).(45, 46). Consequently, IgG glycosylation is determined by the metabolic state of the cell, enabling to adopt IgG glycosylation to altered environmental conditions. While, the regulation of glycosyltransferase expression in B cells is still not fully understood, it clearly plays a key role in various physiological and pathological processes.

1.4 THE Fc GAMMA RECEPTOR FAMILY

FcγRs play a vital role in converting the recognition of antigens by IgG into various cellular immune responses. Various proteoforms of both IgG and FcγR influence this process because they largely determine the mutual affinity. Examples are subclass, allotype and glycosylation variations. The impact of IgG proteoforms has been elaborated on above. FcγRs are classified into activating and inhibitory receptors based on the signaling properties of their intracellular domains. Among activating FcγRs in humans are FcγRI, FcγRIIa, FcγRIIc, and FcγRIIIa, all of which contain an immunoreceptor tyrosine-based activation motif (ITAM). The sole inhibitory receptor is FcγRIIb, which transmits signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM).(47) All FcγRs are type I

transmembrane proteins, except FcγRIIIb, which is linked to the membrane via a glycosylphosphatidylinositol anchor.

Glycosylation of FcγRs can influence their antibody-binding properties, ultimately affecting their subsequent immune cell activation. The N-glycans on FcγRs can vary in their composition, structure, and complexity, depending on the cell type and individual. The FcγRIIIa, for example, has five N-glycan sites, of which mainly Asn162 influences IgG1 binding.(48) In vitro studies have shown that the N-glycans occupying the Asn162 site can affect the binding of FcγRs to antibodies, with different types of glycans having varying effects. FcγRIIIa with an oligomannose structure at Asn162, for example, has been shown to have increased IgG1 binding.(48, 49)

1.5 ABERRANT GLYCOSYLATION OF IMMUNE GLYCOPROTEINS AS A POTENTIAL CLINICAL MARKER

Protein glycosylation is not a DNA-template driven process, but is still partially determined by genetic factors and influenced by the microenvironment of the respective cell which produces a glycoprotein.(45, 50, 51) Any disturbances to this microenvironment due to physiological circumstances, such as disease, can alter the activity of glycosyltransferases and glycosidases. This alteration leads to a subsequent change in the glycosylation profile of immune glycoproteins. Even minor changes in glycosylation can have a profound effect on their effector functions and, in turn, deleterious consequences on both innate and adaptive immune cell activity, affecting health and disease.(52) Therefore, aberrant glycosylation of immune glycoproteins has emerged as a potential clinical marker for disease diagnosis and prognosis. In addition, manipulation of the glycosylation pattern, i.e. glycoengineering, has been incorporated in the antibody development workflow to produce glycoforms with improved therapeutic activity.

Body fluids which are relatively easy to obtain and contain various immune glycoproteins, such as plasma or seminal fluid, represent a promising source of potential clinical markers. A shift in glycosylation of plasma glycoproteins was reported in many different autoimmune and inflammatory diseases, as well as cancers.(53, 54) Based on these associations with total plasma N-glycosylation, a few potential clinical markers have been proposed.(55-59) A glycan-based diagnostic test was recently launched by Helena Biosciences and is used for the diagnosis and prognosis of various liver pathologies.(60)

One of the most extensively characterized plasma-derived glycoproteins is IgG. A reduction in galactosylation of IgG was reported across a wide spectrum of pathological conditions, suggesting that alterations in galactosylation are not a disease-specific process, but rather a general phenomenon having deleterious consequences on the adaptive immune cell activity.(14) Although the size of the drop in IgG glycosylation was associated with disease severity and poorer disease prognosis, the functional relevance of agalactosylated and

asialylated IgG is still not fully delineated. Moreover, the open question is whether aberrant glycosylation is a result of inflammatory milieu or actively drives the pathogenicity of IgG.

In addition to the glycosylation profile of bulk IgG, the glycosylation of antigen-specific IgG has been studied for several diseases. Changes in glycosylation of antigen-specific IgG have been linked to pro-inflammatory processes, disease activity and prognosis.(14, 61) For instance, alloantibodies against human platelet antigens, produced during pregnancy against fetal cells, have a reduced fucosylation which is associated with increased severity of fetal and neonatal alloimmune thrombocytopenia.(62) The biological significance of the absence of core fucose is explained by higher affinity of afucosylated IgG to FcγRIII, transforming moderate into potent mediators of immune defence mechanisms, such as ADCC.(13) Afucosylated IgG has the greatest effect on IgG effector functions, making it a potentially powerful diagnostic target for identifying ongoing inflammatory processes. Its significance is highlighted by the development of a high-throughput (ELISA)-based assay for quantifying antigen-specific IgG Fc fucosylation, a test that could facilitate the translation of glycosylation studies from research to clinical laboratories.(63)

Along with the underlying pathological condition, various population factors including age, sex, body mass index, and pregnancy, can impact the glycosylation profile of a protein, leading to inter-individual variation.(64, 65) To account for these confounding factors in cross-sectional studies, it is essential to have a considerable sample size, include sex- and age-matched control samples, and perform sample randomization. Furthermore, a longitudinal study design can be highly beneficial in exploring glycans as disease markers.

The analysis of protein N-glycosylation is critical for advancing our understanding of the role of glycans in health and disease, and for developing and validating new diagnostic and therapeutic strategies. In addition, the glycosylation analysis targeting human glycoproteins in population studies, must be of sufficient high-throughput to identify specific glycan signatures associated with various pathologies. Although studying immune protein glycosylation is a challenging analytical task, recent advances in analytical methodology facilitated the development of glycosylation analysis.

1.6 Analytical methods for N-glycosylation analysis

Recent advances in several analytical technologies including mass spectrometry (MS), liquid chromatography (LC), capillary electrophoresis (CE), carbohydrate microarrays, and nuclear magnetic resonance spectroscopy have led to the development of approaches which are suitable for both comparatively high-throughput and in-depth N-glycosylation analysis of wide range of immune molecules.(4)

Depending on the research question, protein glycosylation can be assessed at four complementary levels, that is glycan-, glycopeptide-, subunit- and intact-glycoprotein-level.(66) These approaches come with their possibilities and limitations that determine

their specific applications. The glycan-level approach relies on the chemical or enzymatic cleavage of glycans from the glycoproteins present in the sample, e.g., by peptide-*N*-glycosidase F (PNGase F) or Rapid^{TM}PNGaseF in case of N-glycosylation. Cleavage results in a mixture of glycans, which are subsequently labelled with a fluorescent dye and analysed. There are several analytical methods used to analyse protein N-glycosylation. The analysis of glycans in a high-throughput manner is currently possible using three powerful techniques: hydrophilic-interaction ultra-high-performance liquid chromatography with fluorescence detection (HILIC-UHPLC-FLD), multiplex capillary gel electrophoresis with laser-induced detection (xCGE-LIF), matrix-assisted laser ionization mass spectrometry (MALDI-MS).(4) Both HILIC-UPHLC-FLD and xCGE-LIF demonstrate remarkable repeatability and superior performance for low-complexity N-glycans, effectively differentiating their constitutional isomers. On the other hand, MALDI-TOF-MS stands out for its analytical throughout and comprehensive information on sialic acid linkage for all N-glycans. Moreover, this techniques offers more informative results for more complex glycan structures, such as tri- and tetra-antennary species.(67) While this glycan-level approach can yield the most structural information on the glycan, it fails to provide the glycan attachment site or a carrier protein, especially for multiply glycosylated proteins. This information can be retained by using a glycopeptide-level approach, where a glycoprotein is subjected to proteolytic cleavage using, for example, trypsin, endoproteinase GluC or proteinase K. The method of choice for the analysis of glycopeptides is reverse-phase-LC-ESI-MS.(4) MS analysis of the obtained (glyco)peptides, particularly tandem MS analysis, can reveal the glycosylation site, glycan composition and glycan structure. The subunit- and intact-glycoprotein-level approaches, characterize not only glycosylation, but also other post-translational modifications on a protein. While this approach provides information about different proteoforms, it requires high-resolution instruments and lacks information on site-specificity. Each of those approaches characterises glycans to a different level of detail and provides orthogonal information. Thus, a combination of these methods is required for a detailed and comprehensive glycosylation analysis.(68)

Glycosylation analysis can be further distinguished based on the analysed sample. It can be conducted on a single glycoprotein after targeted purification or on a complex mixture of glycoproteins, such as human serum.(69) An analysis of the complete repertoire of glycans has been performed for a range of single glycoproteins, such as IgG, IgA or FcγRIIIb.(70-72) However, this approach is limited since it requires the development of protein-specific purification methods. Nevertheless, new methods are being developed for the purification and analysis of glycoproteins, thereby expanding the selection of single glycoproteins that can be studied, as illustrated in **Chapter 4**. In case of clinical biofluids like serum or seminal plasma, due to the complexity of the sample and the presence of many glycoproteins, the glycan inventory is constructed for all glycoproteins in the sample on the level of released glycans, as illustrated in **Chapter 5** and **6**.

1.6.1 Mass spectrometers

Due to high sensitivity, selectivity and throughput, MS, has become a powerful tool for glycosylation profiling. MS has successfully been employed for the analysis of immune glycoproteins on all levels of characterization.(3, 73-75) MS-based analytical platforms adequately ionize glycans or glycoconjugates, detect their mass-to-charge (*m/z*) ratio and report ions in the form of a mass spectrum that plots the relative abundance of the ions as a function of their *m/z* ratio. It is highly compatible with LC.

The fragile nature of oligosaccharides, glycopeptides, and glycoproteins necessitates the use of soft ionization techniques in mass spectrometric analysis. Electrospray ionization (ESI) is often a method of choice as it avoids fragmentation, allowing the detection of the intact analyte.(76) ESI often advantageously creates ions with multiple charges, especially useful for detecting large masses. Multiple charging of large ions decreases their *m/z* values, making them detectable by mass analysers with a relatively narrow mass range. In the context of glycosylation analysis, ESI is especially suitable for the labile sialylated glycans.

Mass analysers distinguish ions using magnetic and/or electric fields. The performance of a mass analyser is typically characterized by mass range, analysis speed, mass accuracy and resolution. The time-of-flight (TOF) analyser is particularly suitable for high-throughput glycosylation profiling of IgG glycopeptides, due to its superior speed. In TOF analyzers, all particles are accelerated at the same time with the same energy. The resulting velocity of glycans/glycoconjugates depends on their size and charge. For more comprehensive and in-depth analyses, the lates generation of orbitrap instruments has been widely accepted, due to the ultra-high resolution.

Modern mass analysers enable the use of innovative MS hybrid fragmentation strategies. These strategies, arising from the combination and variation of multiple conventional fragmentation techniques, such as collision-induced dissociation (collision-induced dissociation, CID and higher-energy collisional dissociation, HCD) or electron-driven dissociations (electron capture dissociation, ECD and electron transfer dissociation, ETD), have been of great benefit to the field of spectrometry-based glycoproteomics, enabling high-end glycopeptide analysis.(77) Each fragmentation strategy yields distinct types of fragmentation spectra. For example, when applied to a glycopeptide analyte, CID generates B/Y-type glycan fragment ions, making it well-suited for breaking labile glycosidic bounds. On the other hand, ETD produces c/z-type ions and is more suitable for sequencing the peptide backbone. However, when these techniques are combined in the form of hybrid fragmentation, they provide information-reach fragments from both peptide and glycan moiety in a single MS/MS spectrum. This makes it a powerful tool for in-depth analysis of glycopeptides.(78, 79)

1.7 SCOPE

The aim of this thesis is to enhance the understanding of the role of glycosylation in the immune system. To achieve this, we employed high-throughput profiling methods on clinical samples, to investigate the dynamic changes in antibody glycosylation during disease and treatment processes. Furthermore, we strove to develop new methodologies for reliable characterization of N-glycosylation profiles, with the objective of expanding the range of immune glycoproteins being analysed, as well as improving existing methods. Changes in glycosylation of immune glycoproteins are typically associated with alterations in homeostasis, inflammatory processes, and disease progression. By detecting such changes in the glycosylation profiles of immune glycoproteins, researchers can identify disease-specific clinical markers, potentially enabling early diagnosis, personalized treatment, and disease progression monitoring.

The regulation of IgG glycosylation, especially fucosylation, which significantly impacts the ability of IgG to effectively trigger immune responses, remains largely elusive. To gain valuable insights into the special location of these regulatory mechanisms, we investigated the potential role of the spleen in modulating IgG glycosylation. In **Chapter 2**, we evaluated IgG-Fc glycosylation in a cohort of splenectomised individuals to address this question. Our findings reveal a shift in IgG Fc-fucosylation upon splenectomy, supporting a potential role of the spleen in regulating IgG afucosylated responses generated by B cells.

Chapter 3 describes the IgG Fc-glycosylation dynamics in a longitudinal cohort of patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Our findings demonstrate that IgG Fc-fucosylation differs between relapsing and non-relapsing patients already 9 months before the relapse, if a rise in ANCA titers was also observed. These results demonstrate the orthogonality of ANCA-rise and IgG fucosylation and position them as valuable components of a potential multifactor clinical marker platform for disease monitoring in AAV. Moreover, we validated the decrease in sialylation of IgG Fc N-glycans over time as a valuable glycosylation-based clinical marker for the prognosis of AAV relapse.(80)

Despite extensive research in the past decades on mapping the glycosylation profile of antibodies, there has been a lack of glycoproteomic methods for glycosylation mapping of other glycoproteins with crucial immunological functions. In **Chapter 4** of this thesis, we developed a comprehensive LC-MS method for site-specific analysis of FcγRIIIb on primary human cells. The developed methodology demonstrates the ability to identify glycosylation differences between donors, subclasses, allotypes, cells and sites of FcγRIII. Studies on FcγR glycosylation are essential, as they may provide a deeper understanding of this additional layer of regulation of the IgG-FcγR interaction and its (patho-)physiological implications.

In **Chapter 5**, we introduce a novel high-throughput method for chromatographic separation and profiling of plasma and serum N-glycans. This method utilizes hydrophilic interaction liquid chromatography-mass spectrometry and a recently introduced fluorescent label, *Rapi*Fluor-MS. Compared to existing methods, the developed approach allows for more rapid sample preparation. Additionally, the improved method takes advantage of the high FLR and MS sensitivity of *Rapi*Fluor-MS label and thus, offers a highly sensitive, rapid and robust method for detecting biological variability in clinical cohorts. With these advancements, our developed method enhanced the analysis of plasma and serum N-glycans.

Chapter 6 presents the evaluation of seminal plasma N-glycosylation using liquid chromatography-mass spectrometry to evaluate the relationship between environmental exposure and male infertility. This study identified several N-glycans with the potential for biomonitoring of exposure to different environmental factors in men with semen abnormalities.

Finally, **Chapter 7** provides a discussion of the main findings presented in this thesis, contextualizing them with the current literature in the field. Furthermore, this chapter also explores future perspectives and challenges in the analysis of glycosylation in immune molecules.

1.8 REFERENCES

1. Rabinovich GA, van Kooyk Y, Cobb BA. Glycobiology of immune responses. Ann N Y Acad Sci. 2012;1253:1-15.
2. Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al. Essentials of Glycobiology. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. Essentials of Glycobiology. 4th ed. Cold Spring Harbor (NY)2022.
3. Trbojevic-Akmacic I, Lageveen-Kammeijer GSM, Heijs B, Petrovic T, Deris H, Wuhrer M, et al. High-Throughput Glycomic Methods. Chem Rev. 2022;122(20):15865-913.
4. de Haan N, Pucic-Bakovic M, Novokmet M, Falck D, Lageveen-Kammeijer G, Razdorov G, et al. Developments and perspectives in high-throughput protein glycomics: enabling the analysis of thousands of samples. Glycobiology. 2022;32(8):651-63.
5. Petrescu AJ, Milac AL, Petrescu SM, Dwek RA, Wormald MR. Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding. Glycobiology. 2004;14(2):103-14.
6. Lubbers J, Rodriguez E, van Kooyk Y. Modulation of Immune Tolerance via Siglec-Sialic Acid Interactions. Front Immunol. 2018;9:2807.
7. Ereno-Orbea J, Sicard T, Cui H, Mazhab-Jafari MT, Benlekbir S, Guarne A, et al. Molecular basis of human CD22 function and therapeutic targeting. Nat Commun. 2017;8(1):764.
8. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. Nature. 2001;409(6821):733-9.
9. Hennet T, Chui D, Paulson JC, Marth JD. Immune regulation by the ST6Gal sialyltransferase. Proc Natl Acad Sci U S A. 1998;95(8):4504-9.
10. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu Rev Immunol. 2007;25:21-50.
11. Walker MR, Lund J, Thompson KM, Jefferis R. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors. Biochem J. 1989;259(2):347-53.
12. Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sci. 2009;30(7):356-62.
13. Dekkers G, Treffers L, Plomp R, Bentlage AEH, de Boer M, Koeleman CAM, et al. Decoding the Human Immunoglobulin G-Glycan Repertoire Reveals a Spectrum of Fc-Receptor- and Complement-Mediated-Effector Activities. Front Immunol. 2017;8:877.
14. Gudelj I, Lauc G, Pezer M. Immunoglobulin G glycosylation in aging and diseases. Cell Immunol. 2018;333:65-79.
15. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S41-52.
16. Justiz Vaillant AA, Jamal Z, Patel P, Ramphul K. Immunoglobulin. StatPearls. Treasure Island (FL)2022.
17. de Haan N, Falck D, Wuhrer M. Monitoring of immunoglobulin N- and O-glycosylation in health and disease. Glycobiology. 2020;30(4):226-40.
18. Quast I, Peschke B, Lunemann JD. Regulation of antibody effector functions through IgG Fc N-glycosylation. Cell Mol Life Sci. 2017;74(5):837-47.
19. van de Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y. The Emerging Importance of IgG Fab Glycosylation in Immunity. J Immunol. 2016;196(4):1435-41.
20. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 2014;5:520.
21. Ferrante A, Beard LJ, Feldman RG. IgG subclass distribution of antibodies to bacterial and viral antigens. Pediatr Infect Dis J. 1990;9(8 Suppl):S16-24.
22. Pucic M, Knezevic A, Vidic J, Adamczyk B, Novokmet M, Polasek O, et al. High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. Mol Cell Proteomics. 2011;10(10):M111 010090.
23. Anumula KR. Quantitative glycan profiling of normal human plasma derived immunoglobulin and its fragments Fab and Fc. J Immunol Methods. 2012;382(1-2):167-76.
24. Bondt A, Wuhrer M, Kuijper TM, Hazes JM, Dolhain RJ. Fab glycosylation of immunoglobulin G does not associate with improvement of rheumatoid arthritis during pregnancy. Arthritis Res Ther. 2016;18(1):274.
25. Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. J Mol Biol. 2003;325(5):979-89.
26. Nose M, Wigzell H. Biological significance of carbohydrate chains on monoclonal antibodies. Proc Natl Acad Sci U S A. 1983;80(21):6632-6.

27. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem. 2002;277(30):26733-40.
28. Subedi GP, Barb AW. The immunoglobulin G1 N-glycan composition affects binding to each low affinity Fc gamma receptor. MAbs. 2016;8(8):1512-24.
29. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. Proc Natl Acad Sci U S A. 2011;108(31):12669-74.
30. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Nat Rev Immunol. 2013;13(3):176-89.
31. Bayry J, Fournier EM, Maddur MS, Vani J, Wootla B, Siberil S, et al. Intravenous immunoglobulin induces proliferation and immunoglobulin synthesis from B cells of patients with common variable immunodeficiency: a mechanism underlying the beneficial effect of IVIg in primary immunodeficiencies. J Autoimmun. 2011;36(1):9-15.
32. Vattepu R, Sneed SL, Anthony RM. Sialylation as an Important Regulator of Antibody Function. Front Immunol. 2022;13:818736.
33. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, Wang LX, et al. Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. J Clin Invest. 2015;125(11):4160-70.
34. Seeling M, Bruckner C, Nimmerjahn F. Differential antibody glycosylation in autoimmunity: sweet biomarker or modulator of disease activity? Nat Rev Rheumatol. 2017;13(10):621-30.
35. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113(16):3716-25.
36. Pincetic A, Bournazos S, DiLillo DJ, Maamary J, Wang TT, Dahan R, et al. Type I and type II Fc receptors regulate innate and adaptive immunity. Nat Immunol. 2014;15(8):707-16.
37. Temming AR, Dekkers G, van de Bovenkamp FS, Plomp HR, Bentlage AEH, Szittner Z, et al. Human DC-SIGN and CD23 do not interact with human IgG. Sci Rep. 2019;9(1):9995.
38. Nagae M, Kanagawa M, Morita-Matsumoto K, Hanashima S, Kizuka Y, Taniguchi N, et al. Atomic visualization of a flipped-back conformation of bisected glycans bound to specific lectins. Sci Rep. 2016;6:22973.
39. Lippold S, Nicolardi S, Dominguez-Vega E, Heidenreich AK, Vidarsson G, Reusch D, et al. Glycoform-resolved FcγRIIIa affinity chromatography-mass spectrometry. MAbs. 2019;11(7):1191-6.
40. Ferrara C, Brunker P, Suter T, Moser S, Puntener U, Umana P. Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1, 4-N-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. Biotechnol Bioeng. 2006;93(5):851-61.
41. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. Nat Med. 1995;1(3):237-43.
42. van de Geijn FE, de Man YA, Wuhrer M, Willemsen SP, Deelder AM, Hazes JM, et al. Mannose-binding lectin does not explain the course and outcome of pregnancy in rheumatoid arthritis. Arthritis Res Ther. 2011;13(1):R10.
43. Peschke B, Keller CW, Weber P, Quast I, Lunemann JD. Fc-Galactosylation of Human Immunoglobulin Gamma Isotypes Improves C1q Binding and Enhances Complement-Dependent Cytotoxicity. Front Immunol. 2017;8:646.
44. van Osch TLJ, Nouta J, Derksen NIL, van Mierlo G, van der Schoot CE, Wuhrer M, et al. Fc Galactosylation Promotes Hexamerization of Human IgG1, Leading to Enhanced Classical Complement Activation. J Immunol. 2021;207(6):1545-54.
45. Klaric L, Tsepilov YA, Stanton CM, Mangino M, Sikka TT, Esko T, et al. Glycosylation of immunoglobulin G is regulated by a large network of genes pleiotropic with inflammatory diseases. Sci Adv. 2020;6(8):eaax0301.
46. Garcia-Vallejo JJ, van Dijk W, van Die I, Gringhuis SI. Tumor necrosis factor-alpha up-regulates the expression of beta1,4-galactosyltransferase I in primary human endothelial cells by mRNA stabilization. J Biol Chem. 2005;280(13):12676-82.
47. Bournazos S, Gupta A, Ravetch JV. The role of IgG Fc receptors in antibody-dependent enhancement. Nat Rev Immunol. 2020;20(10):633-43.
48. Van Coillie J, Schulz MA, Bentlage AEH, de Haan N, Ye Z, Geerdes DM, et al. Role of N-Glycosylation in FcgammaRIIIa interaction with IgG. Front Immunol. 2022;13:987151.
49. Subedi GP, Barb AW. CD16a with oligomannose-type N-glycans is the only "low-affinity" Fc gamma receptor that binds the IgG crystallizable fragment with high affinity in vitro. J Biol Chem. 2018;293(43):16842-50.

50. Cao Y, Song Z, Guo Z, Zhao X, Gong Y, Zhao K, et al. Cytokines in the Immune Microenvironment Change the Glycosylation of IgG by Regulating Intracellular Glycosyltransferases. Front Immunol. 2021;12:724379.
51. Zaytseva OO, Freidin MB, Keser T, Stambuk J, Ugrina I, Simurina M, et al. Heritability of Human Plasma N-Glycome. J Proteome Res. 2020;19(1):85-91.
52. Reily C, Stewart TJ, Renfrow MB, Novak J. Glycosylation in health and disease. Nat Rev Nephrol. 2019;15(6):346-66.
53. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015;15(9):540-55.
54. Dotz V, Wuhrer M. N-glycome signatures in human plasma: associations with physiology and major diseases. FEBS Lett. 2019;593(21):2966-76.
55. Reiding KR, Ruhaak LR, Uh HW, El Bouhaddani S, van den Akker EB, Plomp R, et al. Human Plasma N-glycosylation as Analyzed by Matrix-Assisted Laser Desorption/Ionization-Fourier Transform Ion Cyclotron Resonance-MS Associates with Markers of Inflammation and Metabolic Health. Mol Cell Proteomics. 2017;16(2):228-42.
56. Clerc F, Novokmet M, Dotz V, Reiding KR, de Haan N, Kammeijer GSM, et al. Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. Gastroenterology. 2018;155(3):829-43.
57. Callewaert N, Van Vlierberghe H, Van Hecke A, Laroy W, Delanghe J, Contreras R. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. Nat Med. 2004;10(4):429-34.
58. Rudman N, Gornik O, Lauc G. Altered N-glycosylation profiles as potential biomarkers and drug targets in diabetes. FEBS Lett. 2019;593(13):1598-615.
59. Juszczak A, Pavic T, Vuckovic F, Bennett AJ, Shah N, Pape Medvidovic E, et al. Plasma Fucosylated Glycans and C-Reactive Protein as Biomarkers of HNF1A-MODY in Young Adult-Onset Nonautoimmune Diabetes. Diabetes Care. 2019;42(1):17-26.
60. Helena Biosciences Europe. Glyco Liver Profile: Helena Biosciences Europe; 2013 [Available from: https://www.helena-biosciences.com/en/clinical-electrophoresis/v8-nexus/tests/glyco-liver-profile/.
61. Oosterhoff JJ, Larsen MD, van der Schoot CE, Vidarsson G. Afucosylated IgG responses in humans - structural clues to the regulation of humoral immunity. Trends Immunol. 2022;43(10):800-14.
62. Sonneveld ME, Natunen S, Sainio S, Koeleman CA, Holst S, Dekkers G, et al. Glycosylation pattern of anti-platelet IgG is stable during pregnancy and predicts clinical outcome in alloimmune thrombocytopenia. Br J Haematol. 2016;174(2):310-20.
63. Sustic T, Van Coillie J, Larsen MD, Derksen NIL, Szittner Z, Nouta J, et al. Immunoassay for quantification of antigen-specific IgG fucosylation. EBioMedicine. 2022;81:104109.
64. Chen G, Wang Y, Qiu L, Qin X, Liu H, Wang X, et al. Human IgG Fc-glycosylation profiling reveals associations with age, sex, female sex hormones and thyroid cancer. J Proteomics. 2012;75(10):2824-34.
65. Kristic J, Vuckovic F, Menni C, Klaric L, Keser T, Beceheli I, et al. Glycans are a novel biomarker of chronological and biological ages. J Gerontol A Biol Sci Med Sci. 2014;69(7):779-89.
66. Ruhaak LR, Xu G, Li Q, Goonatilleke E, Lebrilla CB. Mass Spectrometry Approaches to Glycomic and Glycoproteomic Analyses. Chem Rev. 2018;118(17):7886-930.
67. Reiding KR, Bondt A, Hennig R, Gardner RA, O'Flaherty R, Trbojevic-Akmacic I, et al. High-throughput Serum N-Glycomics: Method Comparison and Application to Study Rheumatoid Arthritis and Pregnancy-associated Changes. Mol Cell Proteomics. 2019;18(1):3-15.
68. Yang Y, Liu F, Franc V, Halim LA, Schellekens H, Heck AJ. Hybrid mass spectrometry approaches in glycoprotein analysis and their usage in scoring biosimilarity. Nat Commun. 2016;7:13397.
69. Pan S, Chen R, Aebersold R, Brentnall TA. Mass spectrometry based glycoproteomics--from a proteomics perspective. Mol Cell Proteomics. 2011;10(1):R110 003251.
70. Wojcik I, Senard T, de Graaf EL, Janssen GMC, de Ru AH, Mohammed Y, et al. Site-Specific Glycosylation Mapping of Fc Gamma Receptor IIIb from Neutrophils of Individual Healthy Donors. Anal Chem. 2020;92(19):13172-81.
71. Novak J, Moldoveanu Z, Julian BA, Raska M, Wyatt RJ, Suzuki Y, et al. Aberrant glycosylation of IgA1 and anti-glycan antibodies in IgA nephropathy: role of mucosal immune system. Adv Otorhinolaryngol. 2011;72:60-3.
72. Bondt A, Selman MH, Deelder AM, Hazes JM, Willemsen SP, Wuhrer M, et al. Association between galactosylation of immunoglobulin G and improvement of rheumatoid arthritis during pregnancy is independent of sialylation. J Proteome Res. 2013;12(10):4522-31.
73. Chen J, Li X, Edmondson A, Meyers GD, Izumi K, Ackermann AM, et al. Increased Clinical Sensitivity and Specificity of Plasma Protein N-Glycan Profiling for Diagnosing Congenital Disorders of Glycosylation by Use of Flow Injection-Electrospray Ionization-Quadrupole Time-of-Flight Mass Spectrometry. Clin Chem. 2019;65(5):653-63.

74. Lippold S, Thavarajah R, Reusch D, Wuhrer M, Nicolardi S. Glycoform analysis of intact erythropoietin by MALDI FT-ICR mass spectrometry. Anal Chim Acta. 2021;1185:339084.
75. Selman MH, Hoffmann M, Zauner G, McDonnell LA, Balog CI, Rapp E, et al. MALDI-TOF-MS analysis of sialylated glycans and glycopeptides using 4-chloro-alpha-cyanocinnamic acid matrix. Proteomics. 2012;12(9):1337-48.
76. Edmond de Hoffmann VS. Mass spectrometry: principles and applications. 3rd edition ed2007.
77. Reiding KR, Bondt A, Franc V, Heck AJR. The benefits of hybrid fragmentation methods for glycoproteomics. Trends in Analytical Chemistry. 2018;108:260-8.
78. Yin H, Zhu J. Methods for quantification of glycopeptides by liquid separation and mass spectrometry. Mass Spectrom Rev. 2023;42(2):887-917.
79. Maliepaard JCL, Damen JMA, Boons GPH, Reiding KR. Glycoproteomics-Compatible MS/MS-Based Quantification of Glycopeptide Isomers. Anal Chem. 2023;95(25):9605-14.
80. Kemna MJ, Plomp R, van Paassen P, Koeleman CAM, Jansen BC, Damoiseaux J, et al. Galactosylation and Sialylation Levels of IgG Predict Relapse in Patients With PR3-ANCA Associated Vasculitis. EBioMedicine. 2017;17:108-18.