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Marija Pezer *Editor*

Antibody Glycosylation

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Marija Pezer
Editor

Antibody Glycosylation

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Editor
Marija Pezer
Glycoscience Research Laboratory
Genos Ltd.
Zagreb, Croatia

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Foreword

Antibodies are one of the main weapons in our arsenal for the eternal war against pathogens. They are an elaborate tool that can specifically recognize foreign structures in our body. This is achieved by site-specific recombination of multiple variants of the V, (D), and J sequences of the variable region followed by somatic hypermutation that induces up to a million times higher rate of mutation in this region during antibody maturation. The fact that a completely bizarre process that, against all evolutionary logic, actually induces mutations has been invented during evolution indicates how important antibodies are for our survival. But binding to antigen is only one part of what antibodies do. After binding to a foreign object, antibodies have to activate proper molecular mechanisms to "deal with" this foreign, non-self object. If this non-self antigen is a pathogenic virus, or a bacterium, it has to be eliminated in the most efficient way. If it is on a cell that may be transformed to a tumour cell, or infected by a virus, the entire cell should be eliminated. But if this foreign antigen is a food we eat, dust, or some antigen in the air, then this antigen should be ignored, and the activation of the immune system should be avoided.

The decision of how to react to a foreign antigen is one of the most complex decisions that have to be made, and these decisions have to be made continuously throughout our lifetime. Alternative glycosylation modulates the execution of these decisions by directing IgG to different receptors and in this way activating different branches of our immune system. Fc glycans are an integral part of the CH2 domain of antibodies and as such represent an integral structural component that participates in the interaction with Fc receptors and other proteins. Attaching a different glycan to the polypeptide backbone changes the structure of the antibody and modifies its affinity for different receptors. The best currently known example is the role of core fucose that acts as a "safety switch" against antibody-dependant cellular cytotoxicity (ADCC) by attenuating binding of IgG to Fc-gamma-receptor IIIA.

Contrary to the polypeptide parts of the antibody that can be changed only by inducing changes in the corresponding genes, glycans are encoded in a complex network of at least several dozen genes that are affected by both epigenetics and the environment. This enables flexible and dynamic regulation of antibody function and

is extensively used to fine-tune our immune system. More than thirty years ago, the initial discovery of changes in the IgG glycome composition in diseases was made and until now over 100,000 different IgG glycomes have been analysed in different diseases and physiological states. Changes in IgG glycosylation are associated with numerous diseases, often even before any other symptoms of the disease are detectable, indicating that they might be a part of molecular pathophysiology leading to the disease. With ageing IgG glycome converts from a composition that suppresses inflammation to an inflammation-promoting glycome that seems to be an underlying risk factor in many cardiometabolic diseases.

Glycosylation is an essential element in the development of different therapeutic monoclonal antibodies, and glycoengineered drugs are already on the market. Inter-individual differences in glycosylation are large and may be an important underlying element for the response or non-response to a given drug, but this is still understudied. Hopefully, the recent progress in analytical methods will enable more studies in this direction, which would help us to better understand functional aspects of inter-individual differences in antibody glycosylation.

The book *Antibody Glycosylation* edited by Marija Pezer and written by an international team of accomplished scientists from academia and industry provides a comprehensive overview of biosynthesis, regulation, functionality, analytics, and applications of immunoglobulin glycosylation. By covering automatization and bioinformatics in high-throughput analytical settings, it provides new perspectives for research and development in the field of therapeutic antibodies, biomarkers, vaccinations, and immunotherapy.

Glycoscience Research Laboratory, Gordan Lauc
Genos Ltd., Zagreb, Croatia

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Editor and Contributors

About the Editor

Marija Pezer received her PhD in molecular biology from the University of Zagreb, Croatia, in 2013. She currently works at Genos Glycoscience Research Laboratory, Croatia, where she is investigating the biomarker potential of immunoglobulin G glycosylation patterns in different pathological and physiological states. The Genos lab has performed over 150,000 total plasma and IgG N-glycome analyses (world's No. 1). She is also the Head of product development for GlycanAge—the first glycan-based test for biological age.

Contributors

Margaret E. Ackerman Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH, USA
Thayer School of Engineering, Dartmouth College, Hanover, NH, USA

Kathirvel Alagesan Max Planck Unit for the Science of Pathogens, Berlin, Germany

Pranay Bharadwaj Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH, USA

Robert Burock glyXera GmbH, Magdeburg, Germany

Samanta Cajic Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

Fabio Dall'Olio Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy

Azra Frkatovic Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Richard A. Gardner Ludger Limited, Culham Science Centre, Abingdon, Oxfordshire, UK

Kathrin Göritzer St. George's University of London, London, UK

Noortje de Haan Copenhagen Center for Glycomics, University of Copenhagen, Copenhagen, Denmark

Siniša Habazin Glycoscience Research Laboratory, Genos Ltd, Zagreb, Croatia

Alyssa L. Hansen Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA

Catherine Hayes Proteome Informatics Group, SIB Swiss Institute of Bioinformatics, Geneva, Switzerland
Computer Science Department, University of Geneva, Geneva, Switzerland

Jenifer L. Hendel Ludger Limited, Culham Science Centre, Abingdon, Oxfordshire, UK

René Hennig Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany
glyXera GmbH, Magdeburg, Germany

Roy Jefferis Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

Sakari Kellokumpu Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland

Toma Keser Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

Elham Khosrowabadi Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland

Lucija Klaric MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK

Marija Klasić Faculty of Science, Division of Molecular Biology, Department of Biology, University of Zagreb, Zagreb, Croatia

Kaitlyn A. Lagattuta Harvard-MIT MD-PhD Program, Harvard Medical School, Boston, MA, USA

Steffen Lippold Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

Frédérique Lisacek Proteome Informatics Group, SIB Swiss Institute of Bioinformatics, Geneva, Switzerland
Computer Science Department, University of Geneva, Geneva, Switzerland
Section of Biology, University of Geneva, Geneva, Switzerland

Nadia Malagolini Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy

Yuka Mimura-Kimura Department of Clinical Research, National Hospital Organization Yamaguchi Ube Medical Center, Ube, Japan

Yusuke Mimura Department of Clinical Research, National Hospital Organization Yamaguchi Ube Medical Center, Ube, Japan

Peter A. Nigrovic Division of Immunology, Boston Children's Hospital, Boston, MA, USA
Division of Rheumatology, Inflammation, and Immunity, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Falk Nimmerjahn Chair of Genetics, Department of Biology, Institute of Genetics, University of Erlangen-Nürnberg, Erlangen, Germany
Medical Immunology Campus Erlangen, Erlangen, Germany

Jan Novak Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

Mislav Novokmet Glycoscience Research Laboratory, Genos Ltd, Zagreb, Croatia

Tea Petrović Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Marija Pezer Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Erdmann Rapp Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany
glyXera GmbH, Magdeburg, Germany

Genadij Razdorov Glycoscience Research Laboratory, Genos Ltd, Zagreb, Croatia

Colin Reily Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

Matthew B. Renfrow Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA

Pauline M. Rudd NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Dublin, Ireland
Bioprocessing Technology Institute, Singapore, Singapore

Alyce Russell Centre for Precision Health, Edith Cowan University, Joondalup, Australia
School of Medical and Health Sciences, Edith Cowan University, Joondalup, Australia

Radka Saldova NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Dublin, Ireland
UCD School of Medicine, College of Health and Agricultural Science, University College Dublin, Dublin, Ireland

Jelena Šimunović Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Daniel I. R. Spencer Ludger Limited, Culham Science Centre, Abingdon, Oxfordshire, UK

Jerko Štambuk Glycoscience Research Laboratory, Genos Ltd, Zagreb, Croatia

Richard Strasser University of Natural Resources and Life Sciences Vienna, Vienna, Austria

Irena Trbojević-Akmačić Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Teemu Viinikangas Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland

Wei Wang Centre for Precision Health, Edith Cowan University, Joondalup, Australia
School of Medical and Health Sciences, Edith Cowan University, Joondalup, Australia

Anja Werner Chair of Genetics, Department of Biology, Institute of Genetics, University of Erlangen-Nürnberg, Erlangen, Germany

Olga O. Zaytseva Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

Vlatka Zoldoš Faculty of Science, Division of Molecular Biology, Department of Biology, University of Zagreb, Zagreb, Croatia

Chapter 1
Micro-Heterogeneity of Antibody Molecules

Yusuke Mimura, Radka Saldova, Yuka Mimura-Kimura, Pauline M. Rudd, and Roy Jefferis

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Y. Mimura (✉) · Y. Mimura-Kimura
Department of Clinical Research, National Hospital Organization Yamaguchi Ube Medical Center, Ube, Japan
e-mail: mimura.yusuke.qy@mail.hosp.go.jp

R. Saldova
NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Blackrock, Co Dublin, Ireland

UCD School of Medicine, College of Health and Agricultural Science, University College Dublin, Belfield, Dublin 4, Ireland

P. M. Rudd
NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Blackrock, Co Dublin, Ireland

Bioprocessing Technology Institute, Singapore, Singapore

R. Jefferis
Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK

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Abstract Therapeutic monoclonal antibodies (mAbs) are mostly of the IgG class and constitute highly efficacious biopharmaceuticals for a wide range of clinical indications. Full-length IgG mAbs are large proteins that are subject to multiple posttranslational modifications (PTMs) during biosynthesis, purification, or storage, resulting in micro-heterogeneity. The production of recombinant mAbs in nonhuman cell lines may result in loss of structural fidelity and the generation of variants having altered stability, biological activities, and/or immunogenic potential. Additionally, even fully human therapeutic mAbs are of unique specificity, by design, and, consequently, of unique structure; therefore, structural elements may be recognized as non-self by individuals within an outbred human population to provoke an anti-therapeutic/anti-drug antibody (ATA/ADA) response. Consequently, regulatory authorities require that the structure of a potential mAb drug product is comprehensively characterized employing state-of-the-art orthogonal analytical technologies; the PTM profile may define a set of critical quality attributes (CQAs) for the drug product that must be maintained, employing quality by design parameters, throughout the lifetime of the drug. Glycosylation of IgG-Fc, at Asn297 on each heavy chain, is an established CQA since its presence and fine structure can have a profound impact on efficacy and safety. The glycoform profile of serum-derived IgG is highly heterogeneous while mAbs produced in mammalian cells in vitro is less heterogeneous and can be "orchestrated" depending on the cell line employed and the culture conditions adopted. Thus, the gross structure and PTM profile of a given mAb, established for the drug substance gaining regulatory approval, have to be maintained for the lifespan of the drug. This review outlines our current understanding of common PTMs detected in mAbs and endogenous IgG and the relationship between a variant's structural attribute and its impact on clinical performance.

Keywords Critical quality attributes · Glycoforms · Glycoproteins · Oligosaccharides · Recombinant antibody therapeutics · Posttranslational modifications

Abbreviations

ADA	Anti-drug antibody
ADCC	Antibody-dependent cellular cytotoxicity
AGE	Advanced glycation end product
APR	Aggregation-prone region
ATA	Anti-therapeutic antibody
CDR	Complementarity-determining region
CHO	Chinese hamster ovary
CQA	Critical quality attribute
FcγR	Receptor for Fc portion of IgG
NeuAc	*N*-acetylneuraminic acid

NeuGc	*N*-glycolylneuraminic acid
PD	Pharmacodynamics
PK	Pharmacokinetics
PTM	Posttranslational modification
QbD	Quality by design

1.1 Introduction

Recombinant monoclonal antibody therapeutics are exemplars of translational medicine and a growing class of biopharmaceuticals (Strohl 2018; Xu et al. 2019; Jefferis 2017b; Lu et al. 2020; Wang et al. 2018). Although the outstanding advantage of mAbs is homogeneity with respect to specificity and affinity, antibody molecules undergo various PTMs during biosynthesis, purification, or storage, and in vivo following administration, resulting in micro-heterogeneity (Figs. 1.1 and 1.2) (Jefferis 2017a, c; Beck and Liu 2019). Micro-heterogeneity of a mAb is a concern for the biopharmaceutical industry because structural modifications can impact efficacy and safety. The sources of heterogeneity identified in early studies included the oligosaccharide attached at each Asn297 residue and N-terminal pyroglutamic acid residues on either the heavy (H) or light (L) chains (Mimura et al. 1995). The development of liquid chromatography/electrospray mass spectrometry and tandem mass spectrometry in the 1990s allowed the identification and quantitation of additional proteoforms due to Met oxidation, Asn deamidation, the presence or absence of H chain C-terminal Lys, etc. (Lewis et al. 1994; Roberts et al. 1995). Recent advances in analytical methods have allowed the identification and localization of additional PTMs in mAbs, both within the complementarity-determining

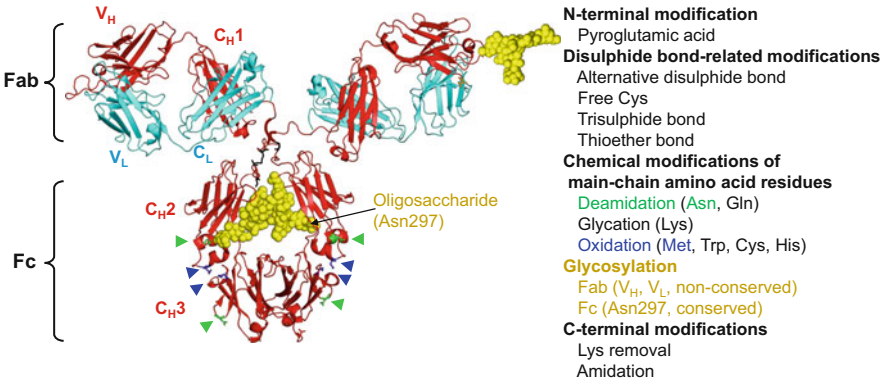

Fig. 1.1 Structure model of a human IgG antibody with the glycans attached to its Fab and Fc regions and common sources of micro-heterogeneity of IgG antibodies. The susceptible sites for oxidation at Met252/Met428 and for deamidation at Asn315/Asn384 are shown by blue and green arrowheads, respectively

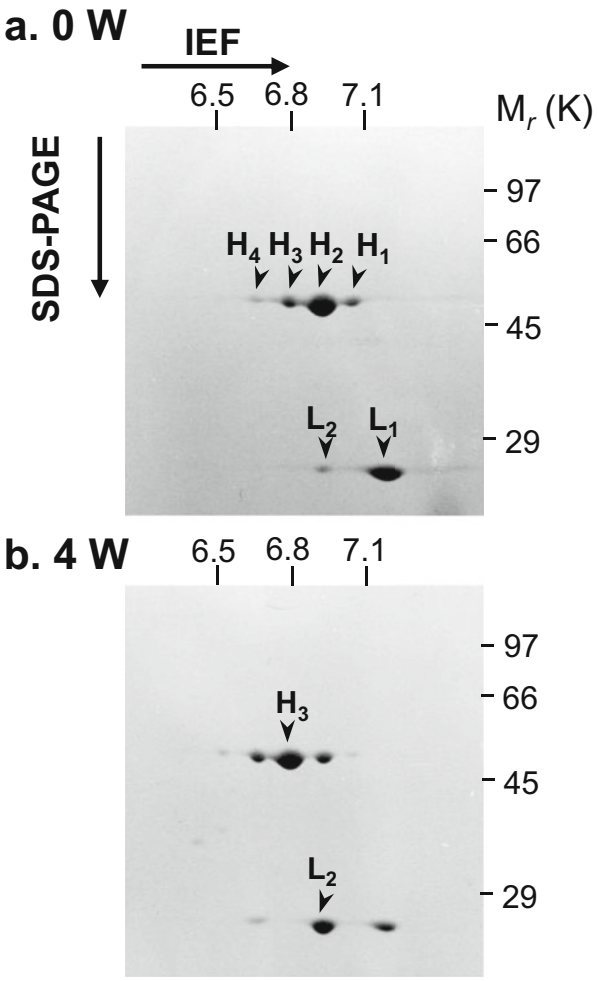

Fig. 1.2 Charge heterogeneity of an IgG1 mAb revealed by 2-D electrophoresis. Before (**a**) and after incubation in 10 mM phosphate-buffered saline at pH 7.0, 37 °C for 4 weeks (**b**). Both the H and L chains exhibit heterogeneity in terms of p*I*, resulting from multiple sources as shown in Fig. 1.1 (**a**). Alterations in the charge properties of the H and L chains took place under physiological conditions (**b**) (Mimura et al. 1998)

regions (CDRs) of the variable (V) regions and within the constant regions, including Asn/Gln deamidation, Asp isomerization, oxidation of Met, Trp, and His, Cys modification, glycation, and Fc and Fab glycosylation (Harris 2005; Beck and Liu 2019; Jefferis 2016; Chicooree et al. 2015; Ambrogelly et al. 2018; Hmiel et al. 2015; Xu et al. 2019). Micro-heterogeneities generated in vivo over the natural lifespan of individual IgG antibody molecules occur at defined amino acid residues and are "recognized" as self by the individual's immune system, resulting in immune tolerance; however, production of mAbs in nonhuman cell lines followed by downstream processing may introduce additional non-self PTMs resulting in loss of structural fidelity, leading to altered stability and biological activities, including the introduction of immunogenic potential.

The addition and processing of complex oligosaccharide moieties within the IgG-Fc is a principal PTM and is defined as a CQA (Jefferis 2017b; Alt et al. 2016; Kepert et al. 2016). Since the glycoform profile of an approved IgG mAb is an essential CQA, they are necessarily produced in mammalian cell lines such as Chinese hamster ovary (CHO) cells and murine plasmacytoma NS0 and SP2/0 cells. However, given that the oligosaccharide biosynthesis process is species- and cell line-specific, and that the observed addition of nonhuman oligosaccharides terminating in α(1–3)-linked galactose (α(1–3)-Gal) or *N*-glycolylneuraminic acid (NeuGc) can result in immunogenicity, the choice and development of a production platform for each mAb constitutes a challenge for the biopharmaceutical industry. One outstanding benefit arising from the above studies has been the demonstration that IgG mAbs bearing oligosaccharides lacking a core fucose residue exhibit increased Fcγ receptor IIIa (FcγRIIIa) binding affinity and consequent increased antibody-dependent cellular cytotoxicity (ADCC) activity; this CQA receives particular attention both within the biopharmaceutical industry and regulators.

Process changes are often introduced within the life cycle of each individual mAb, e.g., optimization for higher productivity and/or increased quality control, etc. All changes must be referred to the regulatory authorities for permission, subject to demonstrating that no adverse responses are experienced by patients. This review outlines the current understanding of common PTMs detected in endogenous IgG and recombinant IgG mAbs and the relationship between a structural variant and its clinical performance.

1.2 Disulfide Bond-Related Modifications

Disulfide bonds result from the coupling of unpaired thiols on Cys residues. The integrity of the disulfide bond is essential to maintain the structure, stability, and biological activities of IgG molecules. The standard structural cartoon for the human IgG1 protein (Eu) exhibits 12 intra-domain disulfide bonds and 4 inter-chain disulfide bonds (Edelman et al. 1969); however, there are variations in the number and pairing of the disulfide bonds between IgG subclasses. IgG1 and IgG4 have two inter-H chain disulfide bonds in the hinge region, while IgG2 and IgG3 have 4 and 11, respectively. For IgG1λ, the L and H chains are connected through the fifth (i.e., the C-terminal) Cys residue of the L chain and the fifth Cys residue of the H chain while for the other IgG subclasses, the C-terminal Cys residue of the L chain is linked to the third Cys residue of the H chain (Liu and May 2012; Lakbub et al. 2016). In addition, there are other Cys-related variants for natural and monoclonal IgG antibodies, including the presence of free thiols, cysteinylation (disulfide bond formation with a free Cys), thioether formation, trisulfide formation, cysteine racemization, and disulfide bond scrambling. Such nonclassical disulfide bond formation can influence the physical stability of IgG and may compromise the safety and efficacy of antibody therapeutics.

Free thiol groups can be detected in natural and recombinant IgG antibodies, although all Cys residues should be involved in disulfide bond formation (–R–S–S–R′–). Free thiols can result from incomplete disulfide bond formation, disulfide bond degradation, and an extra noncanonical Cys residue mostly in the CDRs. Incomplete disulfide bond formation can occur for any intra-domain disulfide bonds and less likely for the inter-chain disulfide bonds (Chumsae et al. 2009). Unpaired Cys residues (Cys 22 and Cys 96) were observed in the V_H domain of a recombinant IgG molecule (Omalizumab, anti-IgE), which substantially reduced its potency (Ouellette et al. 2010; Harris 2005). The unpaired Cys residues were shown to reform a disulfide bond when incubated in serum (Ouellette et al. 2010) or could be prevented by the addition of the oxidizing agent copper sulfate to the cell culture medium (Chaderjian et al. 2005).

Degradation of disulfide bonds can be caused by β-elimination at basic pH, resulting in the generation of dehydroalanine and persulfide. Crosslinking of the resulting Cys and dehydroalanine leads to the formation of a non-reducible thioether linkage (–R–S–R′–), which was found at ~0.4% for recombinant monoclonal IgG1 stored at 4 °C. The L chain—H chain linkage (at L214–H220) was reported to be sensitive for the degradation to the thioether bond formation (Tous et al. 2005), and the rate is higher for IgG1λ than for IgG1κ (Zhang et al. 2013). In addition, the reversibility of the dehydrogenation step can cause racemization of Cys, forming either the L- or D-form (Zhang and Flynn 2013). On the other hand, hydrolysis of the dehydroalanine can also occur, which is an important mechanism that leads to antibody fragmentation in the hinge region.

Insertion of a sulfur atom into a disulfide bond to form trisulfides (–R–S–S–S–R′–) was reported between the H chains of a recombinant human IgG2 (Pristatsky et al. 2009) and later between the L and H chains in all four IgG subclasses and human endogenous IgG (Gu et al. 2010). Trisulfide bonds are formed due to the presence of hydrogen sulfide in the cell culture medium for recombinant mAbs (Kita et al. 2016) and in tissues for endogenous IgG (Gu et al. 2010; Zhao et al. 2001). The presence of trisulfides does not have an adverse effect on antibody function and can be controlled by cysteine feeding in the cell culture medium (Kshirsagar et al. 2012).

Furthermore, an extra noncanonical Cys residue can occur mostly in the CDRs. Cysteinylation of such an unpaired Cys residue is rarely observed in the V regions of IgG, but it can have a significant influence on antibody function (Banks et al. 2008). Cysteinylation and the presence of incomplete disulfide bonds have not been reported in natural human IgGs. Given all the negative impacts of cysteinylation, this modification may have been eliminated from natural IgG during evolution. The same could be true for the presence of a single pair of incomplete disulfide bonds.

A core hinge region sequence of –Cys–Pro–Pro–Cys–, present in IgG1, IgG2, and IgG3, forms a partial helical structure that does not allow for intra-H chain disulfide formation; however, the homologous sequence in the IgG4 subclass is –Cys–Pro–Ser–Cys–, and this does allow for intra-H chain disulfide bond formation. Consequently, natural and recombinant IgG4 antibody populations are a mixture of two hinge isomers, i.e., an inter-chain form with two inter-H chain disulfide bonds

and a second isomer with intra-H chain disulfide bonds that are generated in vivo and in vitro (Rispens et al. 2013).

Additional heterogeneity results from "Fab-arm exchange" with the generation of monovalent bispecific antibody molecules in vivo and in vitro. The exchange is facilitated by the presence of an Arg residue at position 409, in place of the Lys409 present in IgG1, IgG2, IgG3, and a polymorphic variant of IgG4 (Rispens et al. 2013, 2014; Davies et al. 2013). For the Arg409 isoform, lateral noncovalent interactions between the two C_H3 domains are reduced such that, under physiological conditions and in the absence of hinge region inter-H chain disulfide bonds, they dissociate to form HL half-molecules. Re-association is nonselective with the formation of monovalent bispecific antibodies.

Later, alternative disulfide bond linkage in the hinge region of IgG2 has been discovered both in recombinant and in natural human IgG2 (Dillon et al. 2008; Wypych et al. 2008; Martinez et al. 2008). Different IgG2 proteoforms showed a subtle difference in structure and thermal stability (Dillon et al. 2008). Inter-chain disulfide bonds within the IgG2 subclass can form more than one pair of combinations in vivo, namely, the A-, A/B-, and B-forms. The classical inter-chain disulfide bond in IgG2 is termed the A-form, with the Cys near or at the C-terminus of the L chain is linked to the C_H1 domain (the third Cys of the H chain). In the B-form, the C-terminus of the L chain is linked to the hinge region, and both the C_H1 domains have linkages to the hinge region, whereas only one C_H1 domain is linked to the hinge in the A/B-form (Liu and May 2012). This heterogeneity in the disulfide bond formation in IgG2 was first reported for recombinant IgG2 but, later, shown to be present in normal serum-derived IgG2 as well (Wypych et al. 2008; Dillon et al. 2008). The interconversion of these proteoforms was shown to be dynamic and promoted by a reducing environment; such an environment can be provided by the presence of thioredoxin/thioredoxin reductase, released into culture media by effector cells; it can be ameliorated by control of dissolved oxygen levels (Koterba et al. 2012; Hutterer et al. 2013; Rispens et al. 2013, 2014; Davies et al. 2013).

An in vitro model revealed that susceptibility to reduction/oxidation differed between IgG subclasses and L chain types with sensitivity being in the order IgG1λ > IgG1κ > IgG2λ > IgG2κ (Koterba et al. 2012).

1.3 N- and C-Terminal Modifications

1.3.1 N-Terminal Modifications

Gln and its cyclized form, pyroglutamate (pyroGlu) or pyrrolidone carboxylic acid (PCA) are commonly observed at the N-termini of the H and λ L chains of human monoclonal antibodies (Kabat et al. 1991). PyroGlu is also observed in recombinant mAbs (Pang et al. 2015; Dick et al. 2007; Yin et al. 2013). PyroGlu formation involves the cyclization of the N-terminal amine and the subsequent loss of ammonia (−17 Da). Not only Gln but also Glu at the N-terminus of a recombinant mAb are

shown to cyclize to pyroGlu nonenzymatically in vitro (Liu et al. 2011; Chelius et al. 2006). Nearly complete conversion of the N-terminal Gln to pyroGlu was shown to occur spontaneously inside the bioreactor within 15 days, which was affected by buffer composition and temperature and less dependent on pH (Dick et al. 2007). Sodium phosphate, ammonium carbonate buffers, and cell culture media can increase the rate of the reaction, in contrast to water and *Tris*-HCl buffer. On the other hand, the rate of Glu cyclization is much slower and dependent on pH and temperature, with the half-lives of the N-terminal Glu of the L chain of a mAb at 37 °C being 4.8, 19, and 11 months at pH 4.0, 7.0, and 8.0, respectively (Yu et al. 2006). Although the biological significance of pyroGlu formation has not been investigated, the presence of pyroGlu appears to play a role in the stabilization of proteins and peptides, protecting them from chemical and enzymatic degradation. However, as there is no evidence of a specific benefit attached to the presence of N-terminal pyroGlu, to either the H or L chain, it may be considered a CQA and best avoided during clonal selection for a potential mAb therapeutic.

1.3.2 C-Terminal Modifications

C-terminal Lys residue of the H chain of human serum IgG is generally cleaved in vivo to expose the preceding Gly residue (Edelman et al. 1969). The cleavage of the Lys is thought to be catalyzed by an endogenous carboxypeptidase during secretion or in circulation (Harris 1995). When a recombinant human IgG2 antibody was administered in humans, C-terminal Lys was shown to be lost with a half-life of 62 min (Cai et al. 2011). Recombinant IgG molecules produced in mammalian cells exhibit mixed populations of molecules with Lys present or absent on each H chain, and the presence of the Lys results in the formation of basic charge variants. The level of C-terminal Lys can vary between clones of cells, culture conditions with or without serum, and other parameters of the production platform, contributing to charge heterogeneity (Dick et al. 2008; Antes et al. 2007; Jiang et al. 2016).

Although there is no evidence that the presence or absence of C-terminal Lys is a CQA, it presents an issue for quality by design (QbD) (Tang et al. 2013). The absence of C-terminal Lys is presumed to have no impact on the biological activity, pharmacokinetics (PK) and pharmacodynamics (PD), immunogenicity, and safety (Antes et al. 2007). However, taking a conservative approach, potential C-terminal Lys effects on all antibodies cannot be ruled out. It has been reported that the CHO clones expressing a recombinant IgG gene lacking the codon for C-terminal Lys show lower productivities than those with the codon for the Lys and those lacking the codons for the Lys and the preceding Gly (Hu et al. 2017).

Following cleavage of the H chain C-terminal Gly-Lys sequence, amidation of C-terminal Pro residue has been observed for recombinant IgG antibodies (Johnson et al. 2007; Tsubaki et al. 2013). Although the predominant IgG proteoform separated by weak cation exchange high-performance liquid chromatography terminates in Gly residue as the C-termini of both H chains, high-resolution mass spectrometry

of the C-terminal peptide of a minor basic proteoform reveals the presence of amide-terminated Pro (–$CONH_2$) in a single H chain together with carboxylic acid-terminated Gly (–COOH) in the second H chain. C-terminal amidation is catalyzed by peptidyl glycine α-amidating monooxygenase (PAM), and its activity is increased with increasing copper concentrations up to 1 μM in culture media of CHO cells (Kaschak et al. 2011). Pro amidation is considered a common C-terminal modification of the H chain of mAbs, although this modification is undetectable in serum IgG antibodies (Tsubaki et al. 2013). The presence of Pro amidation in mAbs has been shown not to decrease antibody activity, structural stability, in vivo half-life, and subcutaneous bioavailability in rats (Jiang et al. 2016).

1.4 Chemical Modifications of Main-Chain Amino Acid Residues

1.4.1 Deamidation

Deamidation of Asn residues is a major consequence of chemical degradation, contributing to charge heterogeneity of IgG mAbs (Khawli et al. 2010; Wang et al. 2007). Deamidation of Asn residues is observed in vivo, and the position of the Asn residues in the sequence is well defined; however, deamidation during manufacture and storage, etc. may be more randomly distributed (Liu et al. 2008). Deamidation is influenced by the extent of exposure to the external environment (e.g., pH and temperature) and primary structure, with Asn-Gly motif being the most susceptible, followed by Asn-Ser and Asn-Thr motifs. 3-D structures usually impose structural constraints, attenuating deamidation rates (Robinson and Robinson 2001). At neutral to high pH, deamidation of Asn occurs by nucleophilic attack of the backbone nitrogen of the C-terminally flanking amino acid on the side chain carbonyl group of the Asn residue, leading to the formation of a metastable succinimide (cyclic imide) intermediate, which is then hydrolyzed to generate Asp and isoaspartate (isoAsp) in ratios of 1:2–1:3 (Geiger and Clarke 1987).

Deamidation has been detected in normal polyclonal and recombinant IgG molecules at Asn315 and Asn384 of the Fc, with the latter more susceptible (Fig. 1.1, green arrowheads) (Chelius et al. 2005; Liu et al. 2009; Sinha et al. 2009). The relative susceptibility to deamidation at these sites varied between studies; however, the biological significance such as immunogenicity may be ameliorated by the finding that ~23% of normal polyclonal IgG has an Asp384 residue (Liu et al. 2009); thus it may be concluded that healthy humans are constantly exposed to IgG bearing this PTM.

In contrast, deamidation within V regions, particularly within the CDRs, of recombinant antibodies has been shown to compromise antibody specificity and/or binding affinity (Harris et al. 2001; Huang et al. 2005). The kinetics of deamidation at Asn55 in the CDR2 of the H chain of a humanized monoclonal IgG1 antibody was

shown in cynomolgus monkeys, with the half-life of 140 h (Huang et al. 2005). Interestingly, although the recombinant anti-HER2 IgG1 Herceptin has hot spots for spontaneous deamidation at Asn55 in the CDR2 of the H chain and Asn30 in the CDR1 of the L chain under in vitro conditions, the approved drug substance did not exhibit these PTMs; therefore, their presence or absence could be used as a lot release criterion (Harris et al. 2001). In vitro incubations of a mAb in phosphate-buffered saline at pH 7.4 and 37 °C were shown to accurately mimic deamidation taking place in vivo when analyzing the Asn384-containing peptide of the mAb by LC/MS/MS. Thus, deamidation of Asn residues in the V regions can be predicted before the selection of chemically stable antibody candidates (Sydow et al. 2014), and this attribute needs routinely be controlled during manufacturing and storage.

In comparison, Gln residues are relatively resistant to deamidation, and the half-lives of deamidation of synthetic peptides at neutral pH are in the range of 100–5000 days for Gln while in the range of 1–500 days for Asn (Robinson and Robinson 2001). Incubation of a mAb at pH 5–9 at 40 °C for 10 weeks revealed pH dependence for deamidation of Gln, showing that seven Gln residues were susceptible to deamidation at alkaline pH, at Gln13 and Gln82 in the V_H region and Gln366 and Gln422 in the Fc and Gln27, Gln100 and Gln199 in the L chain (Liu et al. 2008). As four of the seven Gln residues that are susceptible to deamidation are located in the V regions, deamidation of these Gln residues can have a significant impact on antibody structure and antigen binding.

1.4.2 Glycation

Glycation is a nonenzymatic condensation reaction between reducing sugars (e.g., glucose) and the primary or the secondary amine (e.g., the ε-amines of Lys residues and N-terminal amines on proteins). Glycation occurs under physiological conditions in natural and recombinant antibodies and could alter their charge property to generate acidic species and their impact on the stability and efficacy, including the aggregation propensity (Wei et al. 2017; Banks et al. 2009). The glycation reaction is initiated by the formation of a Schiff's base between susceptible amines and carbonyls such as aldehydes on reducing sugars, which can then undergo an Amadori rearrangement to form a stable covalent bond between the protein and sugar molecules. Glycation of recombinant therapeutic antibodies can occur due to the exposure to glucose during production in glucose-containing culture media, formulation, and administration in patients (Quan et al. 2008; Fischer et al. 2008; Goetze et al. 2012). Although sucrose used as a stabilizing agent in the formulations may cause glycation due to its hydrolysis into glucose when stored at 37 °C, no evidence of glycation was demonstrated after storage at 2–8 °C for 18 months (Gadgil et al. 2007; Banks et al. 2009). With regard to the extent of glycation, endogenous human Fc contains on average 0.045 glucose/Fc, i.e., ~5% of the glycated species (Goetze et al. 2012), but the glycation levels differ substantially between mAbs and individual batches of the same mAb (Fischer et al. 2008; Miller

et al. 2011). Glycation in the constant regions of IgG1 and IgG2 has been shown not to affect Fc functionality, including the binding to FcγRIIa, FcRn, and staphylococcal protein A (SpA) (Goetze et al. 2012; Mo et al. 2018); however, glycation of Lys located in or adjacent to a CDR should be monitored more carefully. A model recombinant humanized mAb was found to have a highly glycated site at Lys49 (40–60%) of the V_L region when produced in the culture medium containing 0.8% (49.4 mM) glucose (Zhang et al. 2008). The potential decrease in the antigen-binding capacity by glycation in the V regions may be avoided by the selection of an antibody that does not have a Lys residue within the CDRs or by mutation of the Lys to Arg (Miller et al. 2011). The extent of glycation of antibodies can be controlled by modifying glucose feeding strategies and increasing the total concentration of free amino acids and primary amine-containing compounds during cell culture processes (Zhang et al. 2008).

Glycation modifications can further proceed to form advanced glycation end products (AGEs) under oxidative stress, contributing to product coloration. AGE has been identified on recombinant antibodies such as carboxymethyl lysine (Butko et al. 2014). Whether the glycation and AGE modification affects antibody efficacy and safety has yet to be determined, but the presence of AGE has been shown to be immunogenic in rabbits (Ahmad and Moinuddin 2012).

1.4.3 Oxidation

Oxidation of Met to methionine sulfoxide or even methionine sulfone is commonly observed in the Fc of both endogenous and recombinant IgG. It has been demonstrated, for antibodies of the IgG1 and IgG2 subclasses, that Met252 and Met428 residues are prone to oxidation (Chumsae et al. 2007; Bertolotti-Ciarlet et al. 2009; Wang et al. 2011; Pan et al. 2009; Gaza-Bulseco et al. 2008). Although these residues are distant in linear sequence, they are proximal at the C_H2/C_H3 interface (Fig. 1.1, blue arrowheads). Minimal Met252 oxidation levels of 2–5% are reported for purified IgG antibodies in formulation buffers, while lower levels of oxidation are reported for Met428. Oxidation of Met252 and Met428 increases under conditions of accelerated stability testing and on prolonged storage. The interaction site for FcRn that regulates IgG catabolism and placental passage is formed at the C_H2/C_H3 interface, and Met252 and Met428 oxidation has been shown to reduce both the affinity to FcRn and half-life in vivo in mice transgenic for human FcRn (Bertolotti-Ciarlet et al. 2009; Wang et al. 2011). However, it was shown that oxidation of both Met252 and Met428 on both H chains was required to impact IgG1 half-life, and this was only observed when Met252 oxidation was in excess of ~80% (Wang et al. 2011). Both SpA and streptococcal protein G (SpG) bind IgG-Fc at the C_H2/C_H3 interface, and it has been shown that Met oxidation impacts binding affinity for both (Pan et al. 2009; Gaza-Bulseco et al. 2008); therefore, Met252 and Met428 oxidation levels may be a useful CQA and QbD parameter. The impact of Met252 and Met428 oxidation on FcγR binding has been shown to be minimal, but a subtle decrease in

binding to the FcγRIIa-His131 variant was reported (Bertolotti-Ciarlet et al. 2009). Analysis of Herceptin and a potential biosimilar demonstrated that care has to be exercised when resuspending antibody therapeutics since a discrepancy was observed for the level of Met252 oxidation between the innovator product (4.39%) and the proposed biosimilar (10.33%); even so, a note added in proof commented that the value of 4.39% was greater than that determined by the innovator company; no oxidation of Met428 was recorded (Xie et al. 2010). Met oxidation has also been found within CDRs of mouse mAb OKT3 and a human IgG1 mAb (Kroon et al. 1992; Roberts et al. 1995) and may have adverse effects on the potency; therefore, early sequencing and selection for potential antibody clones are employed to eliminate this possibility.

1.5 Aggregation

Control of aggregation of protein pharmaceuticals during manufacture, formulation, storage, and shipping is a major challenge for the biopharmaceutical industry. The level of aggregates in formulated therapeutic antibodies is a CQA as aggregation can often influence the PK/PD of mAbs, adversely impacting safety, efficacy, and the potential for immunogenicity, with the production of ADA/ATA. Protein aggregation is driven by intrinsic factors, including the primary structure, the conformation, and the net charge as well as extrinsic factors, including pH, temperature, protein concentration, ionic strength, and shaking (Li et al. 2016). Various computational algorithms based on the identification of aggregation-prone regions (APRs) in the 3-D protein structure coordinates have been developed to predict the aggregation propensity of a biotherapeutic (Santos et al. 2020). APRs are mainly hydrophobic patches rich in β-branched aliphatic and aromatic residues (Ile, Phe, Val, and Leu) that are exposed to solvent. The propensity for a mAb to aggregate may be determined by the CDRs of the V regions superimposed on an intrinsic susceptibility of the selected H and L chain isotypes. APRs were identified within the C_H1, hinge, C_H2, and C_H3 domains of the IgG1 H chain and the constant regions of both κ and λ L chains (Chennamsetty et al. 2009a). Aggregation-prone motifs in the constant regions of IgG1 are identified in the lower hinge and C_H2/C_H3 interface, including Leu234-Leu235, Met252-Ile253, and Val308-Leu309, which are also important for FcγR and FcRn binding (Wang et al. 2009; Chennamsetty et al. 2009a). The substitution of targeted hydrophobic amino acids with selected hydrophilic residues (e.g., Leu309Lys) has been shown to generate more stable proteins with a diminished tendency to aggregate (Chennamsetty et al. 2009b).

The presence of free thiol is another parameter shown to result in the generation of aggregates through intermolecular disulfide bond formation (Lacy et al. 2008) and is particularly relevant for IgG2 antibodies that contain 50% more free Cys than IgG1 (Huh et al. 2013; Wypych et al. 2008). Additionally, the individual variable sequences can have a profound impact on susceptibilities to aggregation. The germline sequences for V_H, Vκ, and Vλ have been comprehensively reviewed and

analyzed for the aggregation potential of their protein products (Ewert et al. 2003; Rouet et al. 2014). These criteria may now be applied to the selection of clones producing potential IgG therapeutics in the early development process and to the optimization of more soluble and stable IgG therapeutics (van der Kant et al. 2017).

It should be noted that incompatibility of diluents with biopharmaceuticals is observed when mixed with human plasma/serum. Instructions to pharmacists for resuspension of Herceptin and Avastin, for intravenous administration, specified resuspension in 0.9% saline and specifically excluded the use of 5% glucose solutions; no reason was given (Arvinte et al. 2013). The consequences for contravening the instruction demonstrated that titration of either Herceptin or Avastin in 5% glucose into human plasma/serum resulted in the formation of insoluble aggregates. The aggregation was shown to result from isoelectric precipitation of complement proteins (C3, C4, and factor H), fibronectin and apolipoproteins bound to the mAb at pH 6.0–6.2 (Luo and Zhang 2015). It was noted that instructions for resuspension of Remicade specified 0.9% saline, pH 7.2, conditions that did not result in the formation of complexes; similarly, neither did Herceptin or Avastin. Thus, the stability/structure of a mAb therapeutic must be considered beyond that of the formulated drug product as aggregates might form in the patient's bloodstream after its administration in the presence of incompatible diluents, possibly resulting in adverse reactions to patients and/or sensitizing them to later production of ADA.

1.6 Glycosylation

1.6.1 IgG-Fc Oligosaccharide Chain

Glycosylation represents the most frequent posttranslational modification, and it is estimated that ~50% of open reading frame genes encode for proteins that may accept the addition of *N*-linked oligosaccharides at Asn-X-Ser/Thr (where X is any amino acid except Pro) (Jefferis 2017c). IgG has a conserved glycosylation site at Asn297 residue [Eu numbering (Edelman et al. 1969)] of each C_H2 domain of the Fc, and differences in the *N*-linked oligosaccharide are a major source of IgG microheterogeneity (Figs. 1.1, 1.3 and 1.4). Fc glycosylation impacts the stability, function, and safety of therapeutic IgG mAbs and is essential for optimal expression of biological activities mediated through Fcγ receptors and the C1q component of complement (Jefferis 2009; Mimura and Jefferis 2021). IgG-Fc effector functions mediated by Fcγ receptors and C1q, including ADCC, phagocytosis, oxidative burst, and complement-dependent cytotoxicity, are abrogated or severely compromised for aglycosylated or deglycosylated forms of IgG (Nose and Wigzell 1983; Pound et al. 1993; Tao and Morrison 1989). Therefore, the presence of the oligosaccharide attached to Asn297 is a CQA of recombinant antibody therapeutics (Jefferis 2017a, b). The oligosaccharide released from human endogenous IgG-Fc is highly heterogeneous and comprised of the core complex diantennary heptasaccharide ($GlcNAc_2Man_3GlcNAc_2$, designated G0) with the variable addition of fucose,

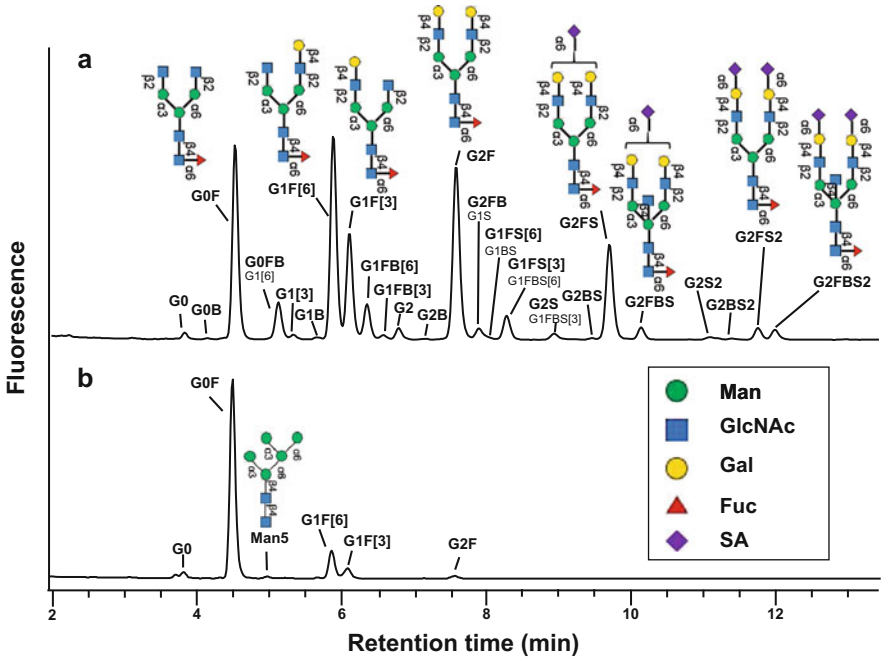

Fig. 1.3 The hydrophilic interaction liquid chromatography profile of oligosaccharides released from (**a**) normal human serum IgG-Fc and (**b**) recombinant IgG1 (bevacizumab). For chromatographic peaks containing multiple oligosaccharide structures, only the predominant components are shown in bold

galactose, bisecting GlcNAc, and *N*-acetylneuraminic (sialic) acid residues (Arnold et al. 2006; Jefferis 2017c) (Figs. 1.3 and 1.4). The heterogeneous oligosaccharides can be classified into three sets (G0, G1, and G2), depending on the number of galactose residues in the outer arms of diantennary oligosaccharides. Within each of these sets are four species that result from the presence or absence of core fucose and bisecting GlcNAc, namely, 16 neutral complex-type structures (Fig. 1.4). Sialylation of the oligosaccharide enhances the heterogeneity, generating 36 possible glycoforms. The oligosaccharides from the Fc fragment of human serum IgG were separated into ~30 structures by hydrophilic interaction liquid chromatography in which fucosylated, monogalactosylated (G1F) glycoforms predominate, with a preference for galactosylation on the α(1–6)-arm (G1F[6]) over the α(1–3)-arm (Fig. 1.3) (Pucic et al. 2011; Mimura et al. 2018). The oligosaccharides of serum IgG-Fc are mostly fucosylated (90–96%), variably galactosylated in aging and diseases and pauci-sialylated (10–20%) (Gudelj et al. 2018; Parekh et al. 1988; Cheng et al. 2020). The outer-arm sugars play important roles in modulating the Fc effector functions, including enhanced ADCC activity of non-fucosylated glycoform. Thus, the rituximab (Rituxan) drug product that gained licensing approval was comprised of ~25% of the G1F oligosaccharide; therefore, regulatory

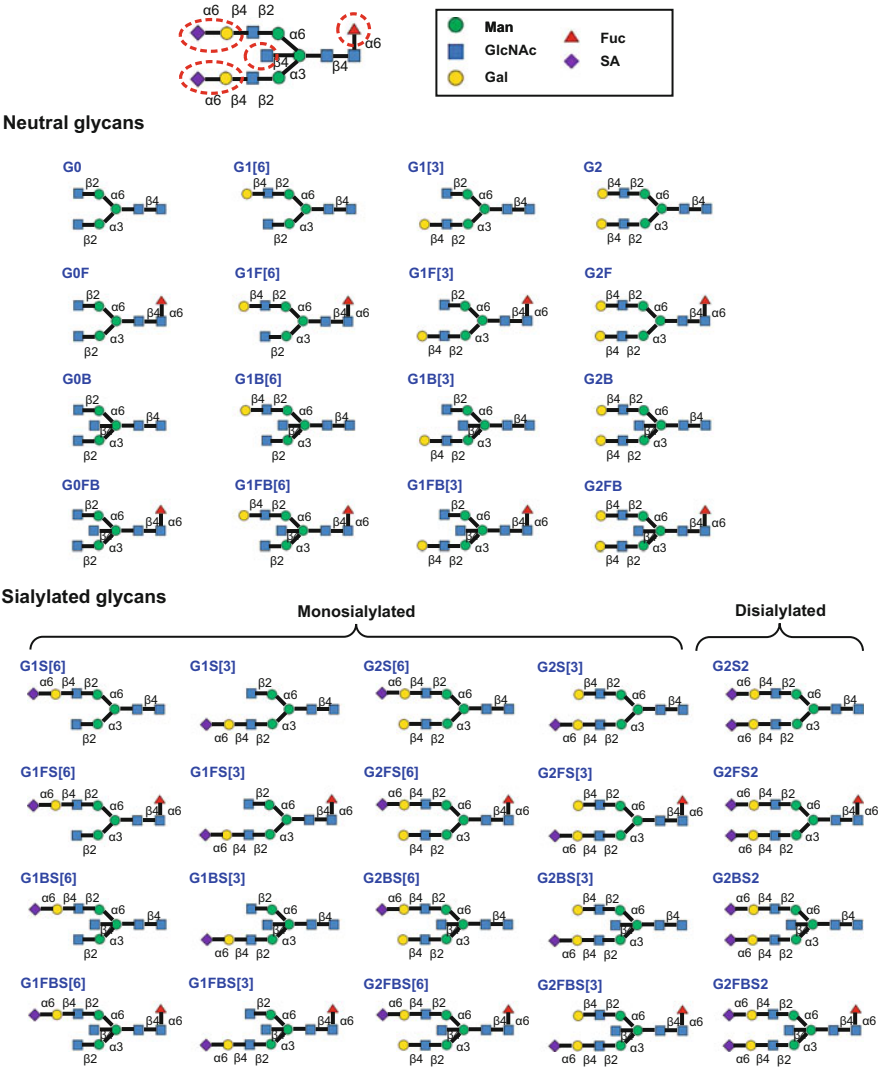

Fig. 1.4 The potential "library" of complex diantennary oligosaccharides associated with human serum IgG-Fc. Heterogeneity arises from variable addition of outer-arm sugars and core fucose (enclosed by red dotted circles) to a "core" heptasaccharide (designated G0)

authorities required that galactosylation of the manufactured product be controlled to within a few percentage points of this value. Analysis of the Fc oligosaccharide of human IgG subclass antibodies revealed unique glycosylation profiles, with preferential galactosylation of the α(1–3)-arm for IgG2 and IgG3 in contrast to that of the α(1–6)-arm for IgG1, IgG4, and polyclonal IgG (Jefferis et al. 1990). The sialic acid of the *N*-glycan of human IgG-Fc is added preferentially on the α(1–3)-arm of the

digalactosylated (G2) glycoforms (van den Eijnden et al. 1980; Grey et al. 1982; Barb et al. 2009). Sialylation occurs in α(2–6)-linkage with *N*-acetylneuraminic acid (NeuAc) in humans, whereas it is in α(2–3)-linkage in CHO-derived recombinant IgG molecules (Takeuchi et al. 1988). Bisecting GlcNAc is present in 10–15% of the *N*-glycans of human IgG-Fc. Currently, licensed therapeutic IgG antibodies are produced in CHO, NS0, and SP2/0 cell lines, and the glycosylation profile of a recombinant glycoprotein is species- and cell type-specific (Raju et al. 2000; de Haan et al. 2020). The oligosaccharide of a CHO-derived therapeutic monoclonal IgG antibody is less heterogeneous than that of human normal polyclonal IgG, with a predominance of non-galactosylated, fucosylated glycoform (G0F) (Fig. 1.3). Bisecting GlcNAc is undetected in recombinant IgG produced in CHO, NS0, and SP2/0 cells (Raju et al. 2000). Terminal α(1–3)-linked galactose (α(1–3)-Gal) and *N*-glycolylneuraminic acid (NeuGc) residues can be attached to the galactosylated oligosaccharides and frequently found in NS0 and SP2/0-derived recombinant IgG antibodies (Sheeley et al. 1997; Stadlmann et al. 2008; Montesino et al. 2012). Such oligosaccharide structures are unnatural and potentially immunogenic in humans. The α-galactosylation and sialylation with NeuGc are reported in cetuximab and infliximab produced from SP2/0 (Qian et al. 2007; Mimura et al. 2009). It has been reported that all humans have IgG antibodies specific for the α(1–3)-Gal epitope (Galili et al. 1993) and that the anti-NeuGc activity is detectable in up to 85% of healthy individuals (Zhu and Hurst 2002; Tangvoranuntakul et al. 2003).

1.6.2 Fab Oligosaccharide Chain

It is established that 15–20% of polyclonal human IgG molecules bear *N*-linked oligosaccharides within the V regions of the κ, λ, or H chains, and sometimes both (Jefferis 2017a). In the immunoglobulin sequence database, ~18% of the V_H sequences contain a potential *N*-linked glycosylation site (Kabat et al. 1991). Very few IgV_H genes have a naturally occurring *N*-linked glycosylation site (Zhu et al. 2002), and the localization of the glycosylation sites in the V_H regions of sequenced proteins is random; therefore, they are likely to be introduced by somatic hypermutation (Dunn-Walters et al. 2000). The functional significance for Fab glycosylation has not been fully evaluated, but studies employing mAbs suggest V region glycosylation can have a neutral, positive, or negative influence on antigen binding (Zhu et al. 2003). On the other hand, in various chronic inflammatory diseases and cancers, including rheumatoid arthritis and follicular lymphoma, high incidence of Fab glycosylation has been observed for anti-citrullinated protein antibodies (ACPAs) (Kempers et al. 2018; Hafkenscheid et al. 2019) and follicular lymphoma-secreted antibodies (Radcliffe et al. 2007; McCann et al. 2008), respectively. The presence of an oligosaccharide in the V region might be associated with the survival of antibody-producing B cells as a consequence of inappropriate activation, selection, and maturation. Analyses of the oligosaccharides of human serum-derived IgG-Fab fragments revealed the presence of diantennary

oligosaccharides showing relatively high levels of galactosylation and sialylation or oligomannose, depending on the location of the glycosylation site (Holland et al. 2006; Hafkenscheid et al. 2017; Radcliffe et al. 2007; Gala and Morrison 2004). The licensed antibody cetuximab expressed in SP2/0 cells is glycosylated at Asn88 of the framework region 3 of the V_H region, bearing complex diantennary, triantennary, and hybrid oligosaccharides (Qian et al. 2007). The Fab oligosaccharide pool contains nonhuman oligosaccharides containing both α(1–3)-Gal (30%) and NeuGc (12%) (Qian et al. 2007) and may induce anaphylactic reaction at least in a proportion of patients due to the presence of IgE antibody targeting α(1–3)-Gal epitope (Chung et al. 2008). The immunogenicity of Fab oligosaccharides is required to be controlled by removing the glycosylation sequon in the V_H and/or V_L or changing or engineering host cell lines to add human-like oligosaccharides. On the other hand, Fab glycosylation may contribute positively to solubility and stability and can be beneficial for improved mAb formulation at higher concentrations (>100 mg/ml). Considering the benefit and disadvantages of Fab glycosylation and the essential demand for product consistency, it offers an additional challenge to the biopharmaceutical industry.

1.7 Conclusion

Antibodies are amongst the most structurally and functionally defined glycoproteins, particularly as potential and/or approved biopharmaceuticals. Advanced orthogonal analytical methods have revealed multiple PTMs and consequent structural micro-heterogeneity. The challenge remains to define the functional diversity of each component within the observed structural micro-heterogeneity and the subsequent impact on therapeutic efficacy and consequent patient benefit. Unintended deviations from the primary amino acid sequence of a biopharmaceutical, as defined by the DNA sequence, must be detected since such products may exhibit modulations of essential functions and/or represent non-self-structures and potential immunogenicity. However, purposeful genetic engineering has been employed for the generation of new/novel constructs expressing a selected profile of effector functions that may be of therapeutic advantage. Micro-heterogeneities at defined residues of a given mAb should be compared with that observed for natural IgG, isolated from human serum, or mAb isolated from the serum of patients following treatment. These data inform the selection amongst potential mAbs being developed as therapeutics and particularly to avoid mAbs having amino acid residues within the CDRs that are susceptible to degradation. During early development, the potential to satisfy established CQAs are assessed and employed to establish comparability between batches and before and after process changes. Thus, a thorough understanding of PTMs of mAbs is critical to the establishment of comparability, successful clinical development, and commercial supply.

The oligosaccharides at the Asn297 sites of the Fc are a CQA, and the biopharmaceutical companies have developed several protocols for the production of

non-fucosylated IgG mAbs, e.g., to enhance ADCC activity for applications in cancer therapy. Further glycoengineering of mAbs is being pursued to develop next-generation mAbs bearing selected homogeneous oligosaccharides with enhanced effector functions; since they are natural glycoforms, this approach does not render them immunogenic. Importantly, while, in general, the association of Fc glycoforms with clinical efficacy has been established, the further impact of sialylation, galactosylation, and fucosylation of IgG mAbs have not been fully elucidated. Long-standing differences have been reported in the literature on whether agalactosylated IgG is proinflammatory whereas sialylated and/or galactosylated IgG are anti-inflammatory in autoimmune diseases; alternatively, why and how anti-RhD antibodies prepared from hyperimmunized healthy anti-D donors have low fucose contents. These and other issues will be discussed in Chap. 15.

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Compliance with Ethical Standards

Conflict of Interest Yusuke Mimura, Radka Saldova, Yuka Mimura-Kimura, Pauline M Rudd and Roy Jefferis declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Part I
Analytical Methods

Chapter 2
Lectin and Liquid Chromatography-Based Methods for Immunoglobulin (G) Glycosylation Analysis

Tea Petrović and Irena Trbojević-Akmačić

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Abstract Immunoglobulin (Ig) glycosylation has been shown to dramatically affect its structure and effector functions. Ig glycosylation changes have been associated with different diseases and show a promising biomarker potential for diagnosis and prognosis of disease advancement. On the other hand, therapeutic biomolecules based on structural and functional features of Igs demand stringent quality control during the production process to ensure their safety and efficacy. Liquid chromatography (LC) and lectin-based methods are routinely used in Ig glycosylation analysis complementary to other analytical methods, e.g., mass spectrometry and capillary electrophoresis. This chapter covers analytical approaches based on LC and lectins used in low- and high-throughput *N*- and *O*-glycosylation analysis of Igs, with the focus on immunoglobulin G (IgG) applications. General principles and practical examples of the most often used LC methods for Ig purification are described, together with typical workflows for *N*- and *O*-glycan analysis on the level of free glycans, glycopeptides, subunits, or intact Igs. Lectin chromatography is

T. Petrović · I. Trbojević-Akmačić (✉)
Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia
e-mail: iakmacic@genos.hr

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a historical approach for the analysis of lectin-carbohydrate interactions and glycoprotein purification but is still being used as a valuable tool in Igs purification and glycan analysis. On the other hand, lectin microarrays have found their application in the rapid screening of glycan profiles on intact proteins.

Keywords Critical quality attribute · Glycosylation analysis · Immunoglobulin · Lectins · Lectin chromatography · Lectin microarrays · Liquid chromatography · Mass spectrometry · *N*-glycans · *O*-glycans

Abbreviations

2-AA	2-aminobenzoic acid
2-AB	2-aminobenzamide
2-PA	2-aminopyridine
2-PB	2-picoline borane
Ab	Antibody
AC	Affinity chromatography
ADCC	Antibody-dependent cell-mediated cytotoxicity
AEX-RP	Anion-exchange-reverse phase
BEH	Bridged ethylene hybrid, stationary phase
CBMs	Malectin carbohydrate-binding modules
CE	Capillary electrophoresis
CF	Chromatofocusing
CGE-LIF	Capillary gel electrophoresis with laser-induced fluorescence detection
Con A	Concanavalin A
CTLD	C type lectin domain
CTLs	C type lectins
DEAE	Diethylaminoethyl
DMB	1,2-diamino-4,5-methylenedioxybenzene
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELLA	Enzyme-linked lectin assay
Endo F	Endoglycosidase F
Endo H	Endoglycosidase H
Endo S	Endoglycosidase S
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcR	Fc receptor
GalNAc	*N*-acetylgalactosamine
GBP	Glycan binding proteins

GFC	Gel filtration chromatography
GlcNAc	*N*-acetylglucosamine
GPC	Gel permeation chromatography
GU	Glucose units
HILIC SPE	Hydrophilic interaction liquid chromatography solid-phase extraction
HLPC	High-performance liquid chromatography
HPAEC	High-performance anion-exchange chromatography
IEX	Ion exchange chromatography
Ig	Immunoglobulin (i.e., IgG = immunoglobulin G; IgA, IgM, IgD, IgE)
IVIg	Intravenous immunoglobulin
LC	Liquid chromatography
LC–ESI-MS	Liquid chromatography electrospray ionization mass spectrometry
mAbs	Monoclonal antibodies
MALDI-TOF–MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBL	Mannan binding lectin
MS	Mass spectrometry
NHS	*N*-hydroxysuccinimide
pAbs	Polyclonal antibodies
PAD	Pulsed amperometric detection
PGC	Porous graphitized carbon
PNGase A	*N*-glycosidase A
PNGase F	*N*-glycosidase F
ProA	Procainamide (4-amino-*N*-[2-(diethylamino)ethyl] benzamide)
QqQ	Triple quadrupole
RP	Reverse phase
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SNA	S*ambucus nigra agglutinin*
TFA	Trifluoroacetic acid
TiO_2	Titanium dioxide
UHPLC-FLR	Ultra-high-performance liquid chromatography with fluorescence detection
ZrO_2	Zirconium dioxide
κ	Kappa
λ	Lambda

2.1 Introduction

Currently, the glycobiology field is working hard to develop new and advance existing rapid, sensitive, and accurate methods for analyzing complex glycan structures in a high-throughput manner. The role of glycans has been studied for decades, and it is known that they play major metabolic, structural, and physical roles in biological systems (Varki et al. 2009). Changes in glycosylation have been observed in aging (Krištić et al. 2014) and different diseases such as alloimmune (Sonneveld et al. 2016; Wuhrer et al. 2009) and autoimmune diseases (Sharapov et al. 2019; Bermingham et al. 2018), cancers (Theodoratou et al. 2016), inflammatory diseases (Trbojevic Akmacic et al. 2015), as well as in infectious diseases (Vadrevu et al. 2018; Larsen et al. 2020), putting glycans into the focus of recent protein and biomarker research. Moreover, the analysis of protein glycosylation has been gaining importance in the pharmaceutical industry since glycans are recognized as critical quality attributes of therapeutic glycoproteins (O'Flaherty et al. 2018).

Glycosylation analysis is still a challenging task, although technological advancements have enabled robust and sensitive glycan analysis on different glycoprotein levels. Several complementary analytical technologies have been routinely used in glycan analysis, and detailed in-depth studies usually require multiple orthogonal approaches (which separate and detect oligosaccharides according to different principles, providing corroboratory qualitative and quantitative information). Liquid chromatography (LC) is a widely applied analytical technique for the detection and purification of (glyco)proteins. One of the fields of LC application is lectin affinity chromatography. It is a common method for the isolation, fractionation, and purification of carbohydrate-containing biomolecules (Mechref et al. 2008). Although LC, capillary electrophoresis (CE), and mass spectrometry (MS) are the most common methods for glycan analysis, lectin-based techniques (e.g., lectin microarrays) can be adopted as a complementary tool for glycan analysis.

2.1.1 Glycosylation

Glycosylation is one of the most common and complex co- and post-translational protein modifications. Glycosylation process, attachment of glycans (oligosaccharides) to proteins or lipids, involves several hundred enzymes, transcriptional factors, and other proteins (Varki et al. 2009). Given the fraction of the mass of the molecule that carries glycan structures, protein glycoconjugates are divided into proteoglycans and glycoproteins. Proteoglycans contain large, branched sugar structures attached to shorter peptide or protein chains, while glycoproteins contain simpler glycans bound to larger protein structures.

The roles of glycoproteins are different. As components of the cell membrane, they participate in cell adhesion and cell recognition, which is especially important in the fertilization process of an egg, embryonic development, and cell

differentiation. Glycan structures serve as signaling molecules for proper protein folding and transport to the appropriate cell compartment. Glycans also play a role in modifying the inflammatory response, which is in numerous studies related to changes in glycosylation of immunoglobulin G (IgG) (Maverakis et al. 2015). Likewise, some pathological conditions are caused by and/or manifested as altered protein glycosylation. Glycans are not excluded from the process of carcinogenesis, metastasis, and autoimmune diseases, due to which they have been the subject of many research studies (Varki et al. 2009).

In addition to DNA, proteins, and lipids, glycans are one of the four basic building blocks of life. They are composed of monosaccharides, which are mostly bound to proteins or lipids in the cellular secretory pathway, i.e., in the endoplasmic reticulum (ER) and the Golgi apparatus (Marek et al. 1999). Glycans on a protein are typically bound by an *N*- or *O*-glycosidic bond. *N*-linked glycans are synthesized by the formation of an *N*-glycosidic bond between the oxygen atom of *N*-acetylglucosamine (GlcNAc) and Asn nitrogen in the protein. The glycan-binding Asn must be a part of the amino acid sequence Asn-X-Ser or Asn-X-Thr, where X may be any of the amino acids except proline. *O*-linked glycans are synthesized by *O*-glycosidic bond formation most commonly from the oxygen atom of the hydroxy amino acid Ser or Thr via *N*-acetylgalactosamine (GalNAc), or much less frequently via GlcNAc, mannose, or fucose. Glycan biosynthesis is covered in detail within the corresponding chapter, so we will not focus on it further.

In addition to the complexity resulting from different types of glycosylation, glycans differ depending on the bond type, branching, composition, and length of the oligosaccharide attached to a protein backbone. Identification and characterization of this enormous complexity and repertoire of possible sugar structures represented and still represents a significant analytical challenge.

2.1.2 Immunoglobulins

Immunoglobulins (Igs), or antibodies (Abs), are our first line of defense against foreign pathogens. In addition to IgG, which is the most abundant Ab in human plasma (de Haan et al. 2017), there are four more classes of Igs: IgM, IgA, IgE, and IgD, distinguishable by the type of their heavy chains (Schroeder and Cavacini 2010). All Igs are composed of two 50–77 kDa class-specific heavy chains that are joined by one or more disulfide bonds. Also, each heavy chain is joined by a disulfide bond to a 25 kDa light chain. There are two types of light chains, called lambda (λ) and kappa (κ). Certain Ig has either κ or λ light chains, never one of each. So far, no differences in function have been found between these chains, and their ratios are different in different animal species. The reason for this variation is unknown (Janeway and Travers 2001). Fab (F stands for fragment; ab—antigen binding) is the antigen recognition and binding domain, whereas the Fc fragment (named Fc because it showed a crystallization tendency) is comprised of the heavy chain region that interacts with Fc receptors (FcRs) on immune cells triggering

different effector functions. While IgM and IgE lack the hinge region and are thus more rigid in the structure, IgG, IgA, and IgD isotypes have a flexible linker containing *N*- and *O*-glycans separating the Fab and Fc regions. Igs are heavily glycosylated, and these glycan modifications are critical for the appropriate function of all Igs.

As stated above, while Fab fragments recognize antigens, the Fc fragment has an effector function, which means that it decides what sequence of actions will follow antigen recognition. The specificity of interactions through the regions of the Fab IgG fragment is employed by exogenous therapeutic monoclonal antibodies (mAbs) used in the treatment of cancer, viral diseases, autoimmune diseases, and many others (Adams and Weiner 2005; Chan and Carter 2010; Scott et al. 2012; Singh et al. 2009; Sliwkowski and Mellman 2013). The Fc fragment interacts with FcR on the immune system effector cell to initiate an appropriate sequence of reactions (Bruhns et al. 2009; Jiang et al. 2011; Nimmerjahn and Ravetch 2008; Siberil et al. 2007). The effector functions of Igs can be very different, even opposite, such as inflammation and suppression of the immune response. Since the amino acid sequence of the Fc fragment, unlike the Fab fragment, is immutable, this cannot be the source of different effector activities. Therefore, *N*-glycans are the ones that enable different responses to antigens (Ahmed et al. 2014).

Glycosylation is of great importance for the proper functioning of the immune system, and one of the most studied glycoproteins is IgG (Rudd et al. 2001). Each CH2 domain of IgG heavy chain carries a covalently bound biantennary *N*-glycan at the evolutionary conserved Asn residue 297 (Butters 2002; Mimura et al. 2018). In addition to Fc *N*-glycans, 15–20% of IgG molecules have glycans in the Fab domain (Stadlmann et al. 2010). These Fab glycosylation sites are not evolutionarily conserved but have resulted from somatic hypermutation during the antigen-specific immune response (Dunn-Walters et al. 2000). The fact that there are no conserved glycosylation sites in the Fab domain and that it is only glycosylated in a fraction of IgG molecules makes Fab glycans more challenging to study.

Bound oligosaccharides have structural and functional importance for IgG and its effector functions (Lauc et al. 2014). For example, on Fc glycans, the lack of galactose residues is associated with rheumatoid arthritis (Parekh et al. 1985). The addition of sialic acid dramatically alters the physiological role of IgG, changing it from a pro-inflammatory to an anti-inflammatory mediator (Böhm et al. 2012). Fucose supplementation to the Fc glycan core impairs the binding of IgG to FcγRIIIa and significantly reduces the antibody-dependent cell-mediated cytotoxicity (ADCC) (Scanlan et al. 2008). Age, as well as gender, correlate with specific patterns of IgG glycosylation (Krištić et al. 2014).

Glycosylation of IgA, as the second most abundant Ab in human serum, has been the most studied after IgG, mostly in IgA nephropathy (Coppo and Amore 2004). IgA glycosylation changes have also been reported in rheumatoid arthritis and pregnancy (Bondt et al. 2017). Both isotypes of IgA—IgA1 and IgA2—contain two conserved *N*-glycan sites per heavy chain, one at Asn263 in the Cα2 domain and one at Asn459, which is the terminal amino acid of its 18-amino acid tail piece. Allotypes of IgA2, IgA2m(1), and IgA2m(2) contain additional evolutionary

conserved *N*-glycan sites. IgA2m(1) contains one *N*-glycosylation site in Cα2 and Cα1 domain, while IgA2m(2) contains one site in Cα2 and two *N*-glycosylation sites in Cα1 domain. Additionally, IgA1 has nine potential *O*-glycosylation sites in the 23-amino acid long, proline-rich hinge region. In serum IgA1, three to five of these *O*-glycosylation sites are occupied (Field et al. 1994). IgA2 is not *O*-glycosylated.

Despite the key role of Ig glycosylation in many physiological and pathological processes (Gudelj et al. 2018), the monitoring of Ig glycosylation is often neglected in clinical and immunological research. Historically, glycosylation analysis in large cohorts has been lagging behind other types of analyses due to the underdeveloped methodology that would enable fast, robust, and affordable profiling of glycans in 100 or 1000 of patient and control samples. However, this has been changing with technological advances and the development of high-throughput methods for glycan analysis using several analytical technologies that provide information on glycosylation sites, site occupancy, and content of glycan variants attached to glycoproteins, mainly ultra-high-performance liquid chromatography with fluorescence detection (UHPLC–FLR), liquid chromatography-electrospray mass spectrometry (LC–ESI–MS), capillary gel electrophoresis with laser-induced fluorescence detection (CGE–LIF), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS) (Huffman et al. 2014). Moreover, lectin-based microarrays have been used to analyze glycan profiles of purified glycoproteins or cell surface proteins (Zhang et al. 2016).

While Ig glycan analysis by MS and CE technologies will be covered in the following chapters, here, we'll describe the robust and sensitive standard approaches in low- and high-throughput *N*-glycosylation analysis of IgG and other Igs based on LC and lectins. Also, new techniques for Ig glycosylation analysis will be mentioned, although some of them are still not routinely used.

2.2 Liquid Chromatography

The most widely used method for the detection and purification of proteins in general is LC. The principle of chromatography is the distribution or partition of individual molecules between two different phases, mobile and stationary phase, based on their relative affinities. Chromatographic techniques can be divided regarding the physical properties of the mobile phase, the nature of the stationary or mobile phase, and the mechanism of separation in the chromatographic system. That is why each method is popularly named after one of its specific features (Wilson and Walker 2010), e.g., ion-exchange chromatography (IEX), chromatofocusing (CF), size-exclusion chromatography (SEC), reverse phase (RP) chromatography, affinity chromatography (AC), etc. (Coskun 2016).

Stationary phases are usually chemically modified small particles packed into a column or a monolith (single piece stationary phases with interconnected large channels). Packed column efficiency depends on the stationary phase particle size, with smaller particles generally resulting in more efficient analyte separation. While

high-performance liquid chromatography (HPLC) column particles are usually 3–5 μm in diameter, ultra-high-performance liquid chromatography (UHPLC) columns are <2 μm in diameter. Consequently, UHPLC columns allow more efficient separation, which is a significant advancement for complex mixture separation (e.g., mixture of glycans), but on the other hand, require higher system pressures to move a mobile phase through the system.

In the following sections, we will cover LC applications for Ig purification and *N*- and *O*-glycan analysis on different levels: free glycans, glycopeptides, glycosylation analysis of Ig subunits, and intact proteins. Currently used LC-based approaches for *N*-glycosylation analysis of Igs are summarized on Fig. 2.1.

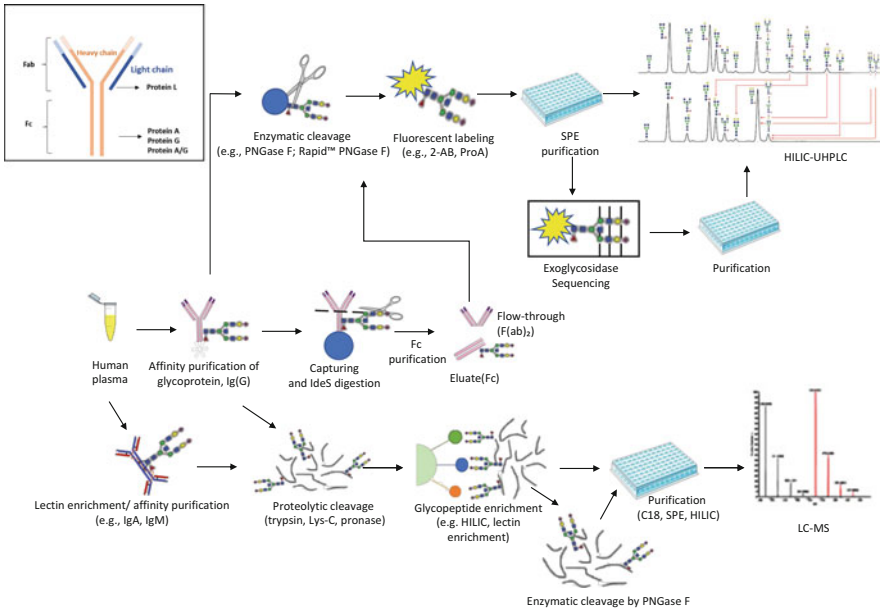

Fig. 2.1 The summary of currently used liquid chromatography-based approaches for N-glycosylation analysis of immunoglobulins (Igs). Free glycans are labeled with a fluorescent dye, e.g., 2-aminobenzamide (2-AB) or procainamide (ProA) and purified or trimmed by exoglycosidases before hydrophilic interaction ultra-high-performance liquid chromatography (HILIC-UHPLC). For analysis of Ig glycosylation on the subunit and whole protein level, IdeS digestion is widely used. Glycopeptide analysis is usually performed using liquid chromatography coupled to mass spectrometry (LC-MS). To obtain glycopeptides, IgG is most often digested with trypsin, enriched with HILIC or lectins, and purified before analysis. Insert: General structure of IgG, with marked heavy and light chains, as well as Fab and Fc fragments. Binding sites for Proteins L, A, and G are shown with arrows

2.2.1 *Immunoglobulin Purification by Liquid Chromatography*

2.2.1.1 Affinity Chromatography

Diverse approaches exist for the purification of Igs from plasma and other biological fluids. Affinity chromatography is the most widely used separation technique for Ab purification due to its ease of use, speed, yield, and specificity, relying on reversible binding between a protein and its cognate ligand (Walters 1985; Ayyar et al. 2012; Hage et al. 2012). Cuatrecasas et al. in 1968, introduced affinity purification, but over the past few decades, considerable efforts were made in terms of selectivity, specificity, reproducibility, product recovery, storage, maintenance, and economy (Cuatrecasas et al. 1968; Arora et al. 2017). To obtain a streamlined purification process, it is necessary to consider the purification procedure, type of ligand, and the matrix to which it is attached, which may require optimization depending on the type/class of Ab and its ability to recognize the immobilized ligand (Arora et al. 2017).

A variety of bacterial proteins are known to bind mammalian Igs, including protein A (Konrad et al. 2011; Nilsson et al. 1987), G (Akerström and Björck 1986), L (Rodrigo et al. 2015), and their recombinant derivatives (fusion proteins); protein LG (Kihlberg et al. 1996), protein LA (Svensson et al. 1998), and protein AG (Ghitescu et al. 1991). Protein A has a high affinity for the Fc region of different Ig isotypes as well as the Fab region of the human VH3 family (Graille et al. 2000; Naomi et al. 2012). Studies showed that protein A does not bind all subclasses of human IgG equally. While it strongly binds subclasses IgG1, IgG2, and IgG4, it only weakly binds subclass IgG3 (Graille et al. 2000; Björck and Kronvall 1984; Walls et al. 2017). Furthermore, protein A can be used to enrich IgD from preparations of other Ig classes since it binds IgG (strongly), IgA, IgM, and IgE (weakly) (Arora et al. 2017; Rodrigo et al. 2015). Protein A can be covalently bonded to a natural (agarose or cellulose) or a synthetic (polyvinyl ether, pore glass, or polymethacrylate) base matrix (Hilbold et al. 2017). Disadvantages of protein A Ig purification are high costs associated with the support, ligand leaching, and caustic instability (Ramos-de-la-Peña et al. 2019). Despite a lot of advantages of using NaOH for resin cleaning, it was shown that NaOH changes the three-dimensional structure of protein A and G and their affinity towards Ig binding (González-Valdez et al. 2014; Naik et al. 2011). To overcome this, several studies have tried to develop enhanced protein A resins as stationary phases tolerating high NaOH concentrations (up to 0.5 M) (Ramos-de-la-Peña et al. 2019; Linhult et al. 2004). Furthermore, different formats (monoliths, membranes, and microspheres) have been tested to carry out the capture of mAbs via protein A, but none of these formats have replaced chromatographic columns as the standard for biotechnology industry. The main drawbacks are elevated resins costs, their limited lifetime compared to other resins and potential regulatory issues. To develop improved cost-effective solutions for Ig purification, alternative ligands to protein A (e.g., aptamers, artificial binding

proteins, engineered Ig-binding proteins, etc.) have been designed and studied (Kruljec and Bratkovič 2017).

Although protein A, protein G, and protein L chromatography are techniques most widely used for the purification of mAbs, protein A chromatography is still considered the golden standard (Hilbold et al. 2017). Furthermore, protein A also interacts with polyclonal antibodies (pAbs) produced by different cell lines with diverse antigen-binding properties (Hilbold et al. 2017). Because of its high binding activity and high purity of isolated Igs, protein A chromatography is commonly used in the pharmaceutical industry (Bolton and Mehta 2016). Although great efforts have been made to decrease the costs and increase the performance of protein A technology, further research is needed to develop and enhance processes, stationary phases, and ligands capable to surpass the simplicity and cost-effectiveness currently offered by protein A-based chromatography.

Protein G and Protein L are ligands with functions similar to that of protein A. However, protein A is not recommended for the isolation of mouse mAbs because it lacks affinity for mouse IgG1, making protein G a more suitable alternative since it binds IgG from most species (Saha et al. 2003). Also, the pH required to dissociate bound IgG is lower, making protein G a commonly used tool for IgG purification. In contrast to protein A, which binds the IgG3 subclass weakly or not at all (depending on the source), protein G binds all subclasses of human IgG equally (GE Healthcare, n.d.). However, protein G has an albumin-binding site which may cause problems with contamination. A recombinant form of protein G that lacks the albumin-binding site is preferred.

Protein L is another Ig-binding protein obtained from the bacteria *Peptostreptococcus magnus* (Myhre and Erntell 1985). It does not bind to the Ig Fc domain but instead interacts with all Ig classes (i.e., IgG, IgM, IgA, IgE, and IgD) with κ light chains (De Chateau et al. 1993), enabling their purification (Rodrigo et al. 2015). Since it binds with high affinity to a large number of Igs with κ1, κ3, and κ4 light chains, protein L is also suitable for the purification of Fab, scFv (short-chain variable region), F(ab)2, and Ab derivatives (Grodzki and Berenstein 2010a). Since protein L binds to a wider range of Igs than either protein A or G, it is a useful tool in affinity chromatography and for Ab immobilization.

To improve the binding characteristics of the bacterial proteins, several fusion proteins were developed. In general, these fusion proteins combine the Ig-binding domains of the three main bacterial Ig-binding proteins: A, G, and L. For instance, protein AG is a recombinant fusion protein that includes four Ig-binding domains from protein A and two from protein G. Protein AG binds to the broadest range of IgG subclasses from human, mouse, and rabbit. It binds to all human IgG subclasses and to IgA, IgE, IgM, and slightly to IgD (Grodzki and Berenstein 2010a).

The major disadvantage of the use of bacterial Fc-binding proteins in chromatographic affinity purification of Abs, in addition to high production costs, is Abs elution in acidic conditions. These low-pH elution conditions may cause some Abs to aggregate or denature and alter the subclass distribution or glycan composition (Ramos-de-la-Peña et al. 2019; Gagnon 1996). Also, low-pH exposure of IgG has been found to cause Cγ2 unfolding associated with protonation of specific acidic

residues (McMahon and O'Kennedy 2000). Furthermore, low-pH IgG exposure induces other conformational changes resulting in increased aggregation and hydrophobicity, which significantly alter FcγR-binding behavior and biological activity (Lopez et al. 2019).

Mannan binding lectin (MBL) and jacalin (extracted from jackfruit) have also been used in the affinity purification of Abs since they specifically bind human IgM and IgA, respectively (Roque-Barreira et al. 1986; Nevens et al. 1992).

2.2.1.2 Melon Gel Chromatography

Besides affinity purification on immobilized protein A, G, or L, Melon gel is a relatively new approach for IgG isolation (Thermo Fisher Scientific, USA). Melon gel, in contrast to protein A and protein G, binds all non-γ-globulin and plasma proteins while allowing purified IgG to be collected in the flow-through fraction. As mentioned before, low pH which is used during IgG immunoaffinity purification using, e.g., protein G can alter its antigen-binding behavior, lead to aggregation and cause denaturation (Nevens et al. 1992; Gagnon et al. 2015; Gagnon and Nian 2016). Furthermore, it is demonstrated that low-pH IgG purification approaches can dramatically alter F(ab')2 antigen recognition (McMahon and O'Kennedy 2000; Djoumerska-Alexieva et al. 2010). Melon gel purification, in contrast, does not require low-pH elution conditions.

Moreover, it was shown that low pH causes IgG aggregation and enhances binding to Fc receptors, impacting the Ab-binding kinetics and affinity, compared to IgG samples purified via Melon gel (Lopez et al. 2019; Dorion-Thibaudeau et al. 2014). It is known that the binding affinity of IgG for the Fc receptors can be modulated by the IgG subclass (Jefferis et al. 1994), and each of the IgG subclasses has a unique binding profile to FcγRs (Vidarsson et al. 2014). Lopez et al. compared protein G and Melon gel purification method in a manner of FcγR binding. For the FcγRIIa and FcγRIIIa receptors, binding affinity follows the hierarchy IgG3 > IgG1 > > IgG2 = IgG4, and it is the strongest for IgG3 in both purification methods. Additionally, IgG1, which follows IgG3 in binding affinity, was significantly more abundant in the Melon gel-purified IgG samples (Lopez et al. 2019).

2.2.1.3 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC) is a liquid column chromatographic technique used for the separation of molecules according to their size when a solution flows through a column filled with porous packing. The method appeared in the late 1950s and was named gel permeation chromatography (GPC) (Moore 1996) or gel filtration chromatography (GFC) (Porath and Flodin 1959). GPC usually refers to the chromatographic separation of synthetic macromolecules with the use of porous gels or rigid inorganic packing particles, while GFC or simply gel filtration refers to a process of biological macromolecules (biopolymers) separation (Kostanski et al.

2004). Size separation is achieved by differential pore permeation. The accessible volume of a pore is greater for a small molecule than for a large one. Because of that, larger molecules have shorter retention times in the pores than smaller ones and are eluted from the column earlier. Over the years, much effort has been spent on the design and manufacture of a wide range of gels compatible with a variety of polymers and mobile phases (Aust et al. 2001). SEC is often used for Ig desalting and buffer exchange, as well as an additional step of purification.

2.2.1.4 Ion-Exchange Chromatography (IEX)

Ion-exchange chromatography (IEX) relies on the binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix (ion exchanger). Such binding is electrostatic and reversible (Grodzki and Berenstein 2010b). IEX can be used for the purification of Igs, but it should be preceded by ammonium sulfate fractionation or followed by affinity chromatography or gel filtration due to its lower selectivity (Grodzki and Berenstein 2010b). Igs can be purified both by cation- (binds positively charged groups) or anion-exchange chromatography (binds negatively charged groups) (Coskun 2016). However, they are most frequently purified by anion-exchange chromatography with diethyl aminoethyl (DEAE) resins (GE Healthcare, n.d.; Grodzki and Berenstein 2010b). The Igs in the sample bind to the ion-exchange matrix and after binding, an equilibration buffer is used to wash the column and remove all molecules that do not bind under the conditions. Elution is done by increasing the ionic strength of the buffer and by changing the pH. At the selected pH, proteins with the lowest net charge will be the first to elute, while the proteins with the highest net charge will be the last to elute. Also, by changing the ionic strength, the proteins bound to the matrix are eluted differentially in a purified and concentrated form (Grodzki and Berenstein 2010b; Yang and Harrison 1996; Fishman and Berg 2019).

2.2.2 N-*Glycan Analysis by Liquid Chromatography*

Liquid chromatography, UHPLC specifically, is being widely used in high-throughput *N*-glycan analysis of isolated glycoproteins (e.g., IgG) and total glycoproteins from plasma/serum samples or tissues because of the relatively low cost of equipment, very good, reliable, and robust quantification, and the ability to separate glycan isomers (Trbojević-Akmačić et al. 2015). The standard procedure for UHPLC analysis includes deglycosylation of proteins, labeling of released glycans with a fluorescent dye, a clean-up procedure to wash out excess reagents, and finally fluorescent detection of labeled and purified glycans.

Additionally, nanoLC systems coupled to MS have been widely used for IgG (Selman et al. 2012; Chandler et al. 2019; Liu et al. 2020), and more recently, IgA analysis (Momčilović et al. 2020) of subclass-specific glycopeptides, where the

chromatographic dimension is utilized to separate glycopeptides into subclass-specific clusters predominantly based on the peptide part. Glycopeptides are further separated and analyzed in MS based on their m/z ratio. This technique is described in more detail in the chapter on MS-based methods for Ig glycome analysis.

2.2.2.1 Glycan Release

N-glycans can be released from glycoproteins using either enzymatic or chemical methods. In contrast to chemical glycan release, enzymatic cleavage of *N*-glycans is more straightforward and specific; this is the reason why it is the most popular way to release *N*-glycans. Several enzymes have been successfully used and optimized for cleaving *N*-linked glycans, such as peptide-*N*-glycosidase F (PNGase F) (Plummer et al. 1984; Tarentino et al. 1985), Rapid™ PNGase F (van de Bovenkamp et al. 2019), peptide-*N*-glycosidase A (PNGase A), endoglycosidases F, H, D (Freeze and Kranz 2010), and S (Collin and Olsén 2001). PNGase F is a widely used glycoamidase due to its broad substrate specificity and high activity. This enzyme will remove oligomannose, hybrid, and complex *N*-glycans attached to Asn by cleaving the bond between a protein and the innermost GlcNAc and converting Asn to Asp. However, it will not remove *N*-glycans with certain modifications of the *N*-glycan core (e.g., α-1,3 fucose) found so far only in slime molds, plants, insects, and parasites (Plummer et al. 1984). It is important to note that although most commercially available PNGase F enzymes today are cloned from *Elizabethkingia miricola* and overexpressed in *Escherichia coli*, different specificities dependent on reaction conditions have been observed (Vilaj et al. 2020). In comparison to PNGase F, PNGase A allows the release of *N*-glycans containing core α-1,3 fucose (Fan and Lee 1997). However, PNGase A shows lower efficiency in *N*-glycan release from glycoproteins (Tarentino and Plummer 1982) and cannot cleave highly complex glycans (Taga et al. 1984). Furthermore, PNGase A is a glycoprotein itself and therefore can be self-deglycosylated, which can bias the outcome of the *N*-glycan analysis by causing contamination from endogenous PNGase A glycan structures (Altmann et al. 1998). Considering the rapid development of *N*-glycoproteome studies, there is a need for PNGases that combine the advantages of both PNGase A and PNGase F (Fan and Lee 1997).

Other used enzymes cleave between the two core GlcNAc residues, leaving one GlcNAc attached to Asn. These endoglycosidases are more specific in terms of the *N*-glycan structures they will cleave. Endoglycosidase H will release oligomannose and hybrid *N*-glycans, but not complex *N*-glycans. Endoglycosidase F will release simple biantennary *N*-glycans, but not oligomannose or hybrid *N*-glycans (Weng et al. 2015). On the other hand, endoglycosidase S (EndoS) has a specific endoglycosidase activity on native IgG by hydrolyzing the glycans attached to the conserved Asn on the heavy chains of IgG (Collin and Olsén 2001; Collin et al. 2002). EndoS hydrolysis has been shown to modulate human IgG/FcγR interactions by influencing the binding/dissociation of IgG to soluble and cell-bound FcγR (Allhorn et al. 2008).

To release glycans, first, it is necessary to denature proteins and break disulfide bonds to make glycans more available to the enzyme. One of the typical approaches is denaturation by high temperature (Akazawa-Ogawa et al. 2018) or sodium dodecyl sulfate (SDS) in combination with β-mercaptoethanol or dithiothreitol (DTT) for reduction of protein disulfide bonds. Moreover, deglycosylation is usually performed at 37 °C for a few hours to overnight to increase the accessibility of glycans to PNGase F and facilitate complete glycan release.

Although denaturation of glycoproteins prior to PNGase F digestion increases deglycosylation efficiency, the digestion process is still time-consuming. Methods such as microwave-assisted deglycosylation have been shown to lead to complete deglycosylation in 10 min (Zhou et al. 2012). In addition, there have been reports on the use of pressure cycling to accelerate enzyme-catalyzed digestion (Szabo et al. 2010). However, these approaches are generally not suited for high-throughput Ig *N*-glycan analyses and may lead to significant errors in quantification due to differences in the release kinetics of *N*-glycans with different structures (Huang and Orlando 2017).

Another approach is a chemical release technique called hydrazinolysis (Patel et al. 1993; Fischler and Orlando 2019). Hydrazinolysis uses anhydrous hydrazine to cleave the complete glycan from the peptide backbone. Unfortunately, the use of hydrazine causes degradation of a peptide backbone and can lead to unwanted modifications of released glycans, such as loss of *N*-acetylation and loss of the free reducing end (Fischler and Orlando 2019). For these reasons, chemical deglycosylation by hydrazinolysis is barely used for glycan analysis.

The main disadvantage of glycan release from whole Ig is the inability to distinguish between Fab and Fc glycans, site-specific glycosylation, and glycosylation originating from different Ig subclasses.

2.2.2.2 Fluorescent Labeling Methods

Labeling of released *N*-glycans plays a crucial role in both detection and characterization by various analytical techniques. Free glycans lack chromophore or fluorophore properties to enable straightforward UV or fluorescent detection; and do not ionize well to result in high-quality mass spectra. Although techniques like high-performance anion-exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD), which do not require glycan derivatization, have been used in the past for glycan analysis (Lee 1990), today glycans are often derivatized during sample preparation, not only to enable their detection but also to enhance the sensitivity of the analysis.

A large number of fluorescent labels have been routinely applied. The easiest way to label released *N*-glycans is via reductive amination, where the amine group of the label reacts with the aldehyde group of a glycan resulting in Schiff base, which is reduced by a reducing agent to yield a secondary amine. In addition to reductive amination, other derivatization methods such as Michael addition, hydrazide labeling, and permethylation have been employed (Ruhaak et al. 2010a).

Many tags have been historically used for the reductive amination of IgG *N*-glycans prior to chromatographic analysis, e.g., 2-aminobenzoic acid (2-AA) (Anumula and Dhume 1998) and 2-aminopyridine (PA) (Takegawa et al. 2006), and some of them, like 2-aminobenzamide (2-AB) (Bigge et al. 1995) and procainamide (4-amino-*N*-[2-(diethylamino)ethyl] benzamide, ProA) (Klapoetke et al. 2010), are still mostly used today.

The label 2-AB has been widely applied in chromatographic analysis of IgG *N*-glycans. Consequently, extensive databases of 2-AB-labeled *N*-glycans have been developed over the years, the most extensive one being GlycoStore (www.glycostore.org) (Klapoetke et al. 2010; Abrahams et al. 2018; Campbell et al. 2008), which contains standardized elution positions of 2-AB- and ProA-labeled *N*-glycans in hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection.

Labeling with 2-AB gives 1:1 stoichiometry, thus allowing relative quantification of different glycans based on fluorescence intensity. It is also very convenient for high-throughput glycan analysis since the sample preparation requires low volumes of solvents, it is relatively fast and simple. Despite these advantages, the major drawback of 2-AB *N*-glycan labeling is poor ionization efficiency, which hinders MS analysis from the same sample making other labels like ProA more attractive.

Having all advantages of 2-AB, ProA additionally shows increased fluorescence and ionization performance (Keser et al. 2018). ProA-labeled glycans are suitable for both (U)HPLC–FLR and ESI–MS analysis, providing more efficient ionization compared to 2-AB, facilitating glycan identification and structure confirmation (Kozak et al. 2015).

Recently, more multifunctional labels, like InstantPC and RapiFluor-MS, which contain both a fluorophore for sensitive chromatographic analysis and an easily ionizable group to facilitate sensitive MS detection, have been developed. InstantPC (instant procaine) rapidly (within 5 min) modifies glycosylamine-bearing *N*-glycans after their enzymatic release, yielding a stable urea linkage. As with ProA, InstantPC contains a tertiary amine to enhance positive mode ESI (Segu et al. 2020). RapiFluor-MS is another commercially available label similar to ProA which is comprised of an *N*-hydroxysuccinimide carbamate reactive group, a quinoline fluorophore, and a basic tertiary amine, an amine that generates a high MS signal in the positive mode and, thus, enhances sensitivity (Lauber et al. 2015). Other "instant" labels used in IgG *N*-glycan analysis are InstantAB (Reusch et al. 2015) and 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Stöckmann et al. 2013).

Advantages of these new commercially available "instant" labels are rapid analysis and increased sensitivity. Labeling can be done in just a few minutes, compared to labeling with the abovementioned traditional labels that have a reaction time of 2–4 h (Stöckmann et al. 2013). Despite these advantages, the major drawback in terms of high-throughput glycan analysis is their price.

2.2.2.3 Reducing Agent

The most used reducing agent in glycan labeling reaction used to be sodium cyanoborohydride, resulting in high yields of labeled oligosaccharides (Bigge et al. 1995). A major drawback of this reagent is that upon hydrolysis, it readily forms the toxic, volatile compound hydrogen cyanide. Today the most used reducing agent is 2-picoline borane (2-PB) (Ruhaak et al. 2010a; Trbojević-Akmačić et al. 2017). 2-PB is an efficient and non-toxic alternative that serves to reductively aminate oligosaccharides with 2-AA, 2-AB, or ProA and is thus less harmful to researchers and the environment, especially considering the analysis of a large number of samples in high-throughput studies. Furthermore, 2-PB can be used in both aqueous and non-aqueous conditions and shows equal efficacies for reductive amination using various fluorescent labels (Ruhaak et al. 2010b). Robust conditions for the reductive amination of IgG *N*-glycans with 2-AB label and 2-PB as the reducing agent that were used in many high-throughput studies were reported in (Trbojević-Akmačić et al. 2017).

Glycan derivatization is enhanced by the addition of glacial acetic acid up to a content of 30% (v/v). Usually, a reaction temperature of 60–65 °C for 2–3 h was found to be optimal. In these conditions, most glycans are derivatized and glycan degradation reactions such as acid-catalyzed loss of sialic acid are minimized.

2.2.2.4 Clean-Up Strategies

After derivatization, labeled glycans have to be purified prior to analysis. Not only does the excess of salts have to be removed (e.g., for MALDI analysis), but also the concentration of remaining free labeling reagent, normally present in large excess during the labeling step, should be reduced. Although different clean-up techniques, like solid-phase extraction (SPE) (Ruhaak et al. 2010b), liquid-liquid extraction (Ciucanu and Kerek 1984), gel filtration (Nakagawa et al. 2007), paper chromatography (Royle et al. 2002), and precipitation (Pabst et al. 2009), have been used for purification, SPE is still the most widely used. Also, SPE is the method of choice for analyzing larger cohorts as it can be adapted to high-throughput setups (Ruhaak et al. 2010b). The diversity of SPE stationary phases and their use in glycan purification is relatively extensive, ranging from RP (Wilhelm et al. 2019), porous graphitized carbon (PGC) (Kolarich et al. 2015; Ashwood et al. 2019), and HILIC (Szabo et al. 2018; Kim et al. 2019), to anion-exchange chromatography (Szabo et al. 2018). A widely applied technique to capture and separate hydrophilic glycans that are released from glycoproteins but not retained by RP chromatography is PGC (Lam et al. 2011). It has successfully been used for the purification of fluorescently labeled glycans and even permethylated glycans (Ruhaak et al. 2010b; Costell et al. 2007). Unfortunately, this glycan-targeting approach has disadvantages due to the high costs of the PGC and the fact that the excess of label may not be removed (Ruhaak et al. 2010b).

The most widely used purification method for the removal of proteins, salts, and the excess of reagents after fluorescent labeling, especially for the analysis of larger cohorts, is HILIC SPE (Trbojević-Akmačić et al. 2016). With this method, excess of label, which is less hydrophilic than glycans, may be removed, and glycans are retained based on their hydrophilic properties (Ruhaak et al. 2008). There is an increasing number of SPE sorbent materials in the market: pipette tips (e.g., ZIPTIP®) (Schmelter et al. 2018; Poole 2003), cartridges (e.g., LudgerClean™T1 cartridge) (Kozak et al. 2015), discs (Poole 2003), filter plates (Berna et al. 2002), magnetics beads (Madhwani and McBain 2016), and sorbent materials (Augusto et al. 2013).

In 2013, an automated platform for high-throughput UHPLC IgG *N*-glycan analysis was developed utilizing an automated liquid handling workstation. That platform allowed the preparation of 96 samples in only 22 h, and its major advantage was rapid and low-cost analysis (Stöckmann et al. 2013). In the meantime, other automatic systems have been successfully used for sample processing in IgG *N*-glycans analysis (Reusch et al. 2014; Reed et al. 2018; Haxo et al. 2016). Nowadays, automated IgG *N*-glycan analysis platforms are increasingly being used to allow 96 samples to be prepared in just 2 h. Moreover, commercially available fast IgG *N*-glycan analysis kits enable rapid enzymatic release and fluorescent labeling of IgG *N*-glycans. Those products facilitate the release of IgG *N*-glycans in minutes. Such methods are faster than conventional protocols and have very good reproducibility.

2.2.2.5 Detection of Labeled Glycans with (U)HPLC

A method that provides robust separation and quantification of fluorescently labeled glycans is HILIC-(U)HPLC. The main advantage of this method over others is the ability to separate structural isomers, as well as neutral and acidic glycans in the same run. The retention time of a glycan depends on its hydrophilic potential, which is influenced by glycan size, charge and structure, and the bonds and branches within the glycan. Analysis of glycans by HILIC is steadily progressing, with the advancements of instrumentation and column chemistry (Mariño et al. 2010). UHPLC over HPLC provides increased resolution and sensitivity due to a stationary phase with a particle diameter of <2 μm, which can function at higher pressures and increased flow rates without decreasing resolution and column efficiency (Ahn et al. 2010a). This reduces the analysis time and mobile phase solvents consumption. The typical mobile phase for HILIC analysis of IgG *N*-glycans is a mixture of acetonitrile and 50 or 100 mM ammonium formate pH 4.4, with glycan separation in a linear gradient of an increasing percentage of the water phase. Ethylene bridged hybrid (BEH) stationary phase has been developed in which methyl silica gel bundles are bridged, ensuring mechanical stability (Alden et al. 2012). The fluorescence detector is equipped with a xenon lamp, and excitation and emission wavelengths are chosen depending on the used fluorescent label to obtain high sensitivity. Dextran hydrolysate, a mixture of labeled glucose oligomers, is used as the external standard for system calibration. The retention time of each oligomer is converted to glucose units

(GU), which are used as reference standard values and for data comparability (Guile et al. 1996). Today, there are bioinformatics tools that contain normalized retention data expressed as GU values. One of those tools is GlycoStore (www.glycostore.org), which contains data on around 850 unique glycan structures (Klapoetke et al. 2010; Abrahams et al. 2018; Campbell et al. 2008). GlycoBase and autoGU (Klapoetke et al. 2010), as well as GlycoProfileAssigner (Duffy and Rudd 2015), are databases and analytical tools developed to assist the interpretation and assignment of HPLC-glycan profiles.

Several orthogonal LC and LC/MS(MS) approaches for the characterization of therapeutic glycoproteins have been recently compared by Largy et al. (2017). It was shown that mixed modes of LC, anion-exchange-reverse phase (AEX-RP), and anion-exchange-hydrophilic interaction liquid chromatography (AEX-HILIC), can be applied for separation of 2-AB and RapiFluor-MS-labeled free *N*-glycans to assess the sialylation profile and as an orthogonal approach to separate *N*-glycans coeluting in HILIC mode (Largy et al. 2017). The mixed-mode AEX-RP column in a gradient of ammonium formate pH 4.5 and acetonitrile enables glycan separation based on their charge (AEX mode) and hydrophobicity (RP mode). Glycans are consequently separated according to their sialylation level, e.g., non-sialylated, monosialylated, disialylated, etc. In contrast to HPAEC-based methods, AEX-RP separation using the abovementioned mobile phases allows straightforward coupling of the LC system to ESI-QTOF-MS, facilitating subsequent MS analysis and glycan structure elucidation. On the other hand, the AEX-HILIC mode enables fast sialylation profiling (13 min run) without MS identification of glycan peaks (Largy et al. 2017). Moreover, RP chromatography has been applied for the quantification of sialic acids after their release in acetic acid and labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) (Largy et al. 2017).

2.2.2.6 *N*-Glycan Sequencing by Exoglycosidases

As mentioned earlier, all human IgGs have *N*-glycans attached to conserved *N*-glycosylation sites in the Fc region, the Asn-297 residues, capped with Man, GlcNAc, Gal, NeuAc (in the α-2,3/6 linkage) or Fuc (α-1,6-linked to the innermost GlcNAc residue—the core fucose). Although *N*-glycan analysis is often performed by using endoglycosidases such as PNGase F and Endo H to cleave off the glycans and identify *N*-glycosylation sites, exoglycosidases are usually employed to cleave monosaccharide units from the nonreducing end of glycans on glycoproteins and glycolipids (Bourne and Henrissat 2001). Sequential *N*-glycan trimming by exoglycosidases has been routinely used in the glycobiology field to obtain structural information about *N*-glycan species (Royle et al. 2006). Application of a set of exoglycosidases results in specific HILIC-(U)HPLC chromatographic glycan peak retention time shifts providing structural information about underlying *N*-glycans. Although exoglycosidase sequencing of fluorescently labeled *N*-glycans is a fast and powerful tool for structural elucidation, not all monosaccharides in a glycan structure are equally accessible to the corresponding glycosidase. For example, several

fucosidases are capable of hydrolyzing core α-1,6-linked fucose, but all of them rely on the previous removal of the majority of the glycan chain (e.g., by endoglycosidases) (Tsai et al. 2017) to enable enzyme binding and core fucose hydrolysis.

Chen et al. recently presented an approach named NGlyoReduction, which trims complex glycans on *N*-glycopeptides using a set of exoglycosidases. Unlike deglycosylation with endoglycosidases, the reduction of glycans generated an oligomannosylated *N*-glycopeptidome, which shares similar retention time and ionization efficiency with intact glycopeptidome (Chen et al. 2016). Oligomannosylated glycopeptidome produced by NGlycoReduction approach can be analyzed by HPLC–ESI–MS to enable the identification of intact glycopeptide structure, peptide sequence, and glycosylation site. The glycan structure of intact glycopeptides can be further identified from their own MS/MS spectra. Advantages of this approach are low cost, simple processing of data, and potential application for characterizing site-specific *N*-glycosylation involving complex *N*-glycans.

2.2.3 O-*Glycan Analysis by Liquid Chromatography*

Analysis of *O*-glycosylation in many cases represents a difficult analytical problem. Contrary to *N*-glycosylation, it is difficult to predict which Ser or Thr presents a potential *O*-glycosylation site just by knowing the amino acid sequence. Moreover, *O*-glycosylation is, in general, far less explored than *N*-glycosylation, mainly due to the underdeveloped methodology for sample preparation. In terms of Ig *O*-glycosylation analysis, another aspect is the fact that only low-abundant human Ig (sub)-classes—IgA1, IgD, and IgG3—contain *O*-glycans (Arnold et al. 2007), making technologies like LC less applicable in Ig *O*-glycan profiling due to its lower sensitivity.

Another aggravating aspect of routine *O*-glycan profiling is the non-existence of specific enzymes that would cleave all types of *O*-glycans. Although an *O*-glycosidase is commercially available, it releases only Core-1 or Core-3 disaccharide *O*-glycans. Recently, the mucin-selective protease StcE has been used for the cleavage of peptide/glycan domains (Malaker et al. 2019). Another enzyme, *O*-endopeptidase, has been used for the digestion of mucin-type glycoproteins and glycopeptides. This enzyme can cleave the N-terminus of *O*-glycosylated Ser or Thr but does not digest at Ser or Thr unless occupied by an *O*-glycan (Yang et al. 2018). However, *O*-endopeptidase is not fully active toward sialylated glycoproteins and therefore, it is suggested that this acidic residue is removed prior to digestion (Yang et al. 2020).

Therefore, a general approach to free *O*-glycan analysis is their release by chemical methods, e.g., β-elimination (Fukuda 1989; Zheng et al. 2009) or hydrazinolysis (Patel et al. 1993). The reaction of β-elimination is usually performed using an ammonia solution. This reaction results in the incorporation of one NH_3 into the amino acid residues to which the glycans are attached. The resulting protein contains a modified amino acid residue with a distinct mass (Zheng et al. 2009;

Rademaker et al. 1998). However, a β-elimination reaction can be difficult to control, is not very robust, and causes side reactions on proteins (Hanisch et al. 2009). It was shown that hydrazinolysis with anhydrous hydrazine at 60 °C for 5 h selectively releases *O*-glycans, while reaction at 95 °C for 4 h releases both *N*- and *O*-glycans (Patel et al. 1993). Released glycans can then be labeled in a reductive amination reaction, e.g., with 2-AB, and analyzed by LC. This approach has been successfully used for *O*-glycan analysis of human secretory IgA (Royle et al. 2003).

Due to analytical challenges in *O*-glycan analysis, most often hyphenated techniques, where a separation technique is coupled with an online spectroscopic detection technology (e.g., LC-MS), have been used for their characterization. Hoffmann et al. (2016) have performed site-specific *O*-glycosylation analysis of Ig glycopeptides in human plasma using RP LC coupled online to ion trap MS after proteinase K digestion, precipitation, and glycopeptide enrichment and fractionation via HILIC. In this study, two *O*-glycopeptides carrying monosialylated T-antigens that might correspond to the J chain were detected and suggested *O*-glycosylation at Thr97. However, IgA1 *O*-glycopeptides have not been detected in this study, most likely due to hydrophilicity of the *O*-glycopeptide region causing these fragments to end up in late eluting HILIC wash fraction or inability to unambiguously identify proteinase K generated glycopeptides. On the other hand, the IgA1 peptide containing the Ser105 *O*-glycosylation site has been detected in a non-glycosylated form. The fact that some other human IgA studies have not been able to detect IgA1 *O*-glycopeptides (Bai et al. 2015) implicates that currently employed *O*-glycan analysis approaches are still not sufficiently sensitive and robust.

The approach by Bai et al. (2015) that enabled novel human IgD Ser121 *O*-glycosylation site identification consisted of albumin depletion using an albumin-affinity column, trypsin digestion, deglycosylation with PNGase F and exoglycosidases, followed by jacalin-affinity-chromatography *O*-glycopeptides enrichment. Collected fractions were then analyzed by LC-MS/MS.

O-glycosylation analysis of therapeutic glycoprotein etanercept (fusion protein of two naturally occurring soluble human TNF receptors linked to an Fc portion of an IgG1), on the level of free *O*-glycans has been successfully performed by their overnight release at 45 °C in sodium hydroxide and sodium borohydride. After neutralization with glacial acetic acid at 0 °C, purification on Dowex 50WX8 hydrogen form column and removal of borates, reduced unlabeled *O*-glycans have been analyzed using PGC column and detected with MS. This alkaline β-elimination reaction in reducing conditions was shown to minimize *O*-glycan peeling, a common problem in *O*-glycan analysis that results in their degradation following release (Largy et al. 2017).

2.2.4 *Liquid Chromatography Coupled to Mass Spectrometry*

Sometimes information obtained in glycan analysis by only using LC is not sufficient to address specific research questions, e.g., glycan structure elucidation/confirmation or site-specific glycan analysis. In these cases, coupling LC to MS system can provide an additional dimension in glycan analysis. Glycan analysis by MS enables highly sensitive high-throughput analysis of the enriched glycoproteome and offers site-specific qualitative and quantitative profiling of glycoproteins, e.g., subclass-specific analysis of IgG Fc *N*-glycans by LC–MS (Selman et al. 2012). On the other hand, it provides mass information of free glycans after their separation in, e.g., PGC or HILIC mode (Ashwood et al. 2019; Vreeker and Wuhrer 2017).

Liquid chromatography allows the separation of isomeric molecules, which then reach the ionization site in the analyzer at different times. Ionization can be performed in several ways: inductively coupled plasma, laser-assisted matrix ionization, chemical ionization, the rapid atomic bombardment of analytes, and ESI. During ionization, the analyte, which was previously in the liquid state, is converted to the ionized gaseous state. After ionization, the analyte enters a mass spectrometer formed by the system quadrupoles (Ho et al. 2003).

Over the past two decades, softer MALDI and ESI techniques (imparting little residual energy onto the analyzed molecule resulting in little fragmentation) have been developed that provide greater sensitivity and generation of intact ions of high-molecular-mass compounds. While ESI often results in doubly charged $[M + 2H]^{2+}$ ions (Wuhrer et al. 2007; Harazono et al. 2008; Rehder et al. 2006) and/or triply charged $[M + 3H]^{3+}$ ions (Wuhrer et al. 2007; Stadlmann et al. 2008; Olivova et al. 2008), MALDI of tryptic IgG glycopeptides typically results in singly charged $[M + H]^{+}$ ions (Takegawa et al. 2006; Kroon et al. 1995). In MS analysis, the ionization efficiency of glycans (especially sialylated glycans) is low, so a suitable derivatization method is required (Mariño et al. 2010). Permethylation (Kang et al. 2008), methyl esterification of sialic acids (Powell and Harvey 1996), or reducing end labeling before MS analysis are typical derivatization methods. To avoid suppression of glycopeptide ionization, it is useful to enrich them from coexisting ionizing peptides.

2.2.4.1 Proteolytic Cleavage

Although recent advances in MS analysis offer many advantages in glycoproteomics, direct analysis of glycoproteins in complex samples by MS is a significant challenge. The low abundance of glycopeptides, the heterogeneity of glycan composition at each glycosylation site, the complexity of glycan structures, and a low ionization are still barriers to overcome (An et al. 2009). The most important steps in Ig glycopeptide LC-MS analysis is the conversion of glycoprotein to glycopeptides and RP clean-up of glycopeptides from the excess reagents. Enzymes such as trypsin, lysyl endopeptidase (Lys-C) (Fernández et al. 2001;

Ongay et al. 2012), endoproteinase Glu-C (V8 protease), and pronase are available for digestion of glycoproteins to glycopeptides (An et al. 2009). Proteolytic cleavage is improved by adding denaturing agents, e.g., guanidine hydrochloride, urea, and ACN (Wuhrer et al. 2007). Also, by using reducing agents such as DTT and β-mercaptoethanol, the accessibility of the proteolytic cleavage site is further improved.

Trypsin is the most widely used proteolytic enzyme in proteomics and glycoproteomics. Trypsin is a serine protease that specifically cleaves proteins at the carboxyl side of Lys and Arg in the preferred mass range for MS, producing interpretable peptide fragmentation mass spectra. Trypsin digestion is generally done at 37 °C overnight in a reaction buffer of 10–100 mM (Tris-HCl or ammonium bicarbonate, pH 8) (Vaezzadeh et al. 2010). As mentioned earlier, denaturing agents such as guanidine-HCl or urea are used to improve trypsin digestion (Wuhrer et al. 2007).

IgG trypsin digestion results in specific peptide moieties for each IgG subclass allowing subclass-specific glycopeptide analysis: EEQYNSTYR for IgG1, EEQFNSTFR for IgG2, and EEQFNSTYR for IgG4. Due to allotype variation in the amino acid at the position N-terminal of the Asn297, a tryptic digest of IgG3 results in a mass identical to either IgG2 peptide (EEQFNSTFR; predominant in Caucasian populations) or IgG4 peptide (EEQYNSTFR; predominant in Asian and African populations) (Dard et al. 2001). Although trypsin digestion is the standard approach for IgG Fc glycopeptide analysis, it was shown that incomplete IgG denaturation and digestion could cause biases in obtained glycopeptide profiles (Falck et al. 2015).

Lyc C is an alkaline protease that cleaves peptides on the carboxyl side of Lys. As for IgG, during a short digestion time and with lower protease/protein ratios, partial cleavage occurs, while almost complete digestion is achieved overnight by digestion at 37 °C and 1:200–1:20 protease/protein ratio (Hirayama et al. 1998).

Endoproteinase Glu-C is a serine protease, which cleaves peptide bonds at the carboxyl-terminal side of Glu. Depending on the pH of the digestion buffer, peptide bonds are further cleaved at the terminal carboxylic side of Asp. Also, endoproteinase Glu-C digestion of human IgG enables the characterization and quantification of plasma-purified IgG1 and IgG4 by LC–ESI–MS and LC–ESI–MS/MS (Huhn et al. 2009).

Pronase is a commercially available mixture of endo- and exopeptidases, nonspecifically cleaving proteins into their individual amino acids. Pronase was applied to elucidate the structural and heterogeneity data of IgG glycans (Rothman et al. 1989).

2.2.4.2 Glycopeptide and Glycan Enrichment

One of the major challenges in the field of MS glycan analysis is the relatively low abundance of specific glycopeptides (Peterman and Mulholland 2006). This can be

alleviated by selective enrichment of glycopeptides from peptide mixtures before MS detection.

Accordingly, various glycoprotein enrichment methods have been developed and discussed in several reviews (Chen et al. 2014). These developed methods are based on HILIC (Selman et al. 2011), SPE (Pezer et al. 2016), and titanium dioxide affinity chromatography (Larsen et al. 2007). Moreover, lectin affinity chromatography is widely used to enrich glycoproteins/glycopeptides (Chen et al. 2017). Lectins will be described in the corresponding section.

Enrichment by HILIC has been commonly used because it is relatively cheap and easy to operate. Different HILIC materials have been used for glycopeptide enrichment. Chemistry of the chosen HILIC stationary phase, composition of the mobile phase, and the properties of the sample itself can affect the efficiency of enrichment (Jensen et al. 2013). Also, SPE enrichment techniques are generally used because they are cheap, simpler than gel-based techniques, and feasible for designing high-throughput MS-based analytical systems. One method of negatively charged sialylated glycopeptides enrichment is chelation interaction using titanium dioxide (TiO_2), zirconium dioxide (ZrO_2), and hybrid metal oxide-based materials, which also allow the purification of neutral glycopeptides (Palmisano et al. 2012; Wan et al. 2011). Hydrophilic interactions between the metal surface and glycopeptides play an important role in the enrichment of neutral glycopeptides (Kayili et al. 2019).

2.2.4.3 Glycopeptide and Glycan Analysis by LC-MS

One of the widely applied analytical techniques for glycopeptide analysis involves a direct analysis of proteolytic digests by RP-HPLC coupled to ESI–MS (Wuhrer et al. 2009). Separation of proteolytic IgG digests by RP-HPLC is mostly performed on a C18 analytical column, but as an alternative to RP-HPLC, graphitized carbon chromatography can be applied (Wagner-Rousset et al. 2008). Trifluoroacetic acid (TFA) or formic acid (FA) are commonly used mobile phase additives (Wuhrer et al. 2007; Harazono et al. 2008; Huang et al. 2005). Although TFA has a positive influence on analyte retention, it can form a gas-phase ion that potentially increases the ESI suppression (Chakraborty and Berger 2005). Therefore, FA is used for the analysis of IgG glycosylation. nanoLC-RP-ESI-ion trap (IT)-MS has been successfully used for *O*-glycopeptide analysis of IgG3 hinge region after proteinase K and trypsin in-gel digestion of protein bands (Plomp et al. 2015).

Another powerful technique for the separation and analysis of glycopeptides is HILIC coupled to ESI–MS, HILIC-ESI–MS. Retention in HILIC is mainly achieved via hydrophilic interactions between the stationary phase and the glycan moieties of the glycopeptides, which result in the separation of glycopeptides from peptides. Separation of glycopeptides can be achieved according to the degree of sialylation (Boersema et al. 2008).

Free mAb *N*-glycans have been successfully analyzed using HILIC-UPLC-QTOF-MS setup after labeling with $^{12}C_6$ and $^{13}C_6$ stable isotope analogs (Δmass = 6 Da) of 2-AA (Millán Martín and Iglesias 2015). This kind of a twoplex

method is ideally suited for comparability studies of mAbs (e.g., lot-to-lot analysis, or innovator and biosimilar similarity assessment), enabling quantitative structural characterization of glycans, including isomeric species.

2.2.4.4 Analysis of Ig Glycosylation on the Subunit and Whole Protein Level

Contrary to bottom-up methods, where proteins are subjected to proteolytic digestion and characterized by MS based on their amino acid sequences and post-translational modifications, middle-up and middle-down approaches rely on the analysis of larger fragments or subunits, e.g., heavy and light Ig chains. These techniques are highly sensitive and informative and reduce sample preparation time and data complexity. Stationary phase based on wide-pore (300 Å) hybrid silica bonded with amide ligand was introduced in 2016 and successfully utilized to resolve glycoforms at the middle-up level of trastuzumab analysis (fragments of 25–100 kDa) after cleavage with IdeS protease (immunoglobulin-degrading enzyme of *Streptococcus pyogenes*) (Periat et al. 2016). Enzyme IdeS is a cysteine protease that cleaves all IgG subclasses at the hinge region resulting in F(ab')2 and Fc fragments and after reduction of disulfide bonds in six 25 kDa domains (a duplicate of each—LC, Fd, and Fc/2). Biochemical properties of this highly specific protease have been studied by Vincents et al. (2004). Additionally, after domain separation, IdeS allows Fab- and Fc-specific released glycan analysis (Anumula 2012) as already described in the previous sections. HILIC separation of Ig fragments using the abovementioned wide-pore stationary phase has been most effective with TFA ion-pairing instead of FA or ammonium formate (usually used in HILIC separation of free glycans). The addition of TFA reduces protein retention and improves resolution and peak shape. Chromatographic resolution can also be improved by the use of several in-line columns (Periat et al. 2016). Since mobile phases containing TFA are volatile, this mode of separation can easily be coupled to MS. Major drawbacks of this approach are required enzymatic digestion and chemical reactions before analysis, allowing the possibility of artifact formation, and limited analysis of protein modifications due to partial sequence coverage (percentage of the protein detected as peptides) (Fornelli et al. 2014). Larger precursor mass makes MS fragmentation and data processing more challenging (Lermyte et al. 2019).

Another approach, not suffering from disadvantages related to sample preparation reactions, is a top-down approach, enabling fast analysis of intact glycoproteins. However, this approach also has a limitation of lower sequence coverage, as well as poor detection of low-abundant glycoforms. The top-down approach is orthogonal to other MS methods based on protein proteolysis. Low-abundant mAb glycoforms were successfully analyzed on intact Ab level using high-resolution nanoLC-chip/MS(/MS) technology (Jacobs et al. 2016). The advantage of this approach is the ability to detect hypoglycosylation (the lack of glycans), the possibility of high-throughput applications due to minimal sample processing, and the quantitative

analysis of glycans on each IgG glycosylation site. This technology can easily be employed for application in lot-to-lot glycoform heterogeneity evaluation of therapeutic Abs, while on the other hand, it is less suitable (compared to subunit analysis) for more detailed characterization due to poorer separation of same molecular weight glycans. To the best of our knowledge, these techniques have been applied so far only for Fc-glycosylation profiling.

2.3 Lectin Techniques

Lectins are glycan-binding proteins (GBPs) that selectively recognize glycan epitopes of carbohydrates or glycoproteins through the reversible binding between the anomeric hydroxyl groups on carbohydrates and the hydrophilic groups from amino acid residues in lectin protein (Hang et al. 2015; Pröpster et al. 2016). This heterogeneous group of proteins with at least one non-catalytic domain reversibly binds to specific accessible glycans present on glycoproteins and glycolipids without altering the structure of the carbohydrates (Lannoo and Van Damme 2014; Wu and Liu 2019). Lectins were initially discovered in plants, and they have been the most extensively studied. Moreover, they were found in other organisms, ranging from viruses (Van Breedam et al. 2014) to humans (Wesener et al. 2017).

Most lectins are multivalent and capable of agglutinating cells and thus are frequently designated as agglutinins. The first alternative name originated from the ability of proteins or glycoproteins to agglutinate the blood group ABO (H) glycotopes (epitopes) and sialic acid on red blood cells (Lannoo and Van Damme 2014). Lectins can be divided into classes based on their amino acid sequences and biochemical properties: C-type lectins (CTLs, require calcium ions for carbohydrate binding); G-type lectins (*Galanthus nivalis* agglutinin-related lectins); L-type lectins (Legume seeds and LysMs in bacterial autolysins); malectin carbohydrate-binding modules (CBMs) (bind glucose oligomers; maltose) (Berg and Tymoczko 2002; Bellande et al. 2017). While G-type and L-type lectin families are mostly found in plants (Bellande et al. 2017), LysM, CBMs are spread in bacteria (Bellande et al. 2017), and C-type lectins are spread in animals (Berg and Tymoczko 2002).

CTLs are the largest family of known GBPs, which comprises 16 different groups, defined by their phylogenetic relationships and domain structures (Drickamer et al. 2002; Zelensky and Gready 2005; Mayer et al. 2017). During evolution, CTLs have interacted with a large range of glycan ligands, although some also bind proteins, lipids, and inorganic molecules. CTLs include selectins, collectins, proteoglycans with C-type lectin domain (CTLD), and endocytic receptors (Cummings and McEver 2009). Selectins are divided into L-selectins (CD62L), E-selectins (CD62E) and P-selectins (CD162) (André et al. 2015; McEver 2015). While L-selectins are expressed by leukocytes, P-selectins are predominantly expressed by platelets and endothelial cells, and E-selectins are found on endothelial cells. These proteins function as adhesion and signaling receptors in many pathways,

including homeostasis and innate immunity, and are crucial in inflammatory responses and leukocyte and platelet trafficking (Mayer et al. 2017).

Collagen-containing C-type lectins, known as collectins, are oligomeric proteins that are characterized by a collagen-like domain with a short Cys rich N-terminus (Drickamer et al. 1986). So far, nine CTLs have been discovered, but MBL was the first one, and it is the most characterized collectin to date (Van De Wetering et al. 2004; Howard et al. 2018). Collectins stimulate in vitro phagocytosis by recognizing surface glycans on pathogens, stimulate the production of cytokines and reactive oxygen species by immune cells, and act before the induction of an Ab-mediated response (Cummings and McEver 2009).

Among the lectin receptors in innate immunity, siglecs and galectins are well described (Manning et al. 2017; Bhide and Colley 2017). While galectins bind β-galactose-containing glycoconjugates, siglecs represent cell surface proteins that bind sialic acid (Pillai et al. 2012).

Proteoglycans with CTLD (lecticans or hyalectins) include aggregan, brevican, versican, and neurocan and they exist in the extracellular matrix (ECM) (Yanagishita 1993; Schmitt 2016). Like the selectins, each of these core proteins contains a CTLD, an epidermal growth factor (EGF)-like domain, and a complement-regulatory protein domain. They mediate leukocyte–endothelium adhesion through various carbohydrate ligands (Nelson et al. 1995; Cummings and McEver 2009; Yamaguchi 2000). Recent studies have shown that the CTLD of aggrecan activates classical and, to a lesser extent, the alternative pathway of complement (Melin Fürst et al. 2013).

Lectins play many key roles in the control of various physiological and pathological processes in living organisms, including cell migration, inflammation, immune defense, fertilization, embryogenesis, infection, and cancer formation (Sharon and Lis 1989, 2003). Also, they mediate cell-cell interactions by binding with complementary carbohydrates on opposing cells (Cummings and McEver 2009; André et al. 2015; Sharon and Lis 1989). Given the vast complexity and diversity of glycan structures, as well as different types of interactions with proteins, it is not surprising that the range and respective biological activities of lectins are substantial. Conveniently, this glycan-recognition and -binding properties have been used as a tool in a wide range of glycoscience applications: detection, isolation of glycoproteins, mapping of neuronal glyco-functions, investigation of carbohydrates on cells and subcellular organelles, selection of lectin-resistant mutants, etc. (Gabius et al. 2011).

Lectins found in nature are mostly purified directly from different organisms. On the other hand, lectins can also be produced by recombinant techniques. To this date, the most reported applications of recombinant lectins are in cancer diagnosis and as anti-microbial, anti-viral, and anti-insect molecules. Furthermore, lectins have been used in the field of functional as well as structural glycomics (Wu and Liu 2019; Oliveira et al. 2013; Hu et al. 2015). The sensitivity of lectins makes them a valuable tool in disease diagnosis compared to instrumental techniques (Pihíková et al. 2015). Additionally, in glycoconjugate analyses where the amount of purified glycoprotein is insufficient for instrumental techniques, including mass spectrometry, techniques

relying on lectin-glycan recognition can be very helpful. The choice of proper lectin for such studies and its quality as a tool highly depends on well-defined lectin specificity. Here we will cover some applications of lectins as stationary phases in chromatography techniques used for Ig *N*- and *O*-glycan analysis, as well as their utilization in increasingly used microarrays.

Lectin analytical methods are based on lectin-carbohydrate interactions and can be divided into traditional and modern ones. Precipitation analysis, originally developed for the detection of antigen-Ab complexes (Wu et al. 1997) and hemagglutination inhibition, based on a lectin-mediated cell agglutination process (Sano and Ogawa 2014), belong to the traditional methods. Today, lectins are used in several immunoassay-like techniques, including enzyme-linked lectin assay (ELLA), lectin-western blot, lectin affinity chromatography, lectin microarrays, biosensor technologies, and as a tool to analyze glycoconjugates (Lauc et al. 2002). Since lectins do not share a common structural feature, there is no universal secondary Abs to lectins. Therefore, to be used in immunoassay-like techniques lectins must be labeled with a suitable tag, e.g., biotin, digoxigenin, or digoxin, which will enable their identification and detection (Lauc et al. 2002).

2.3.1 Lectin Chromatography

One of the main fields of lectin application is lectin affinity chromatography, first developed by Donnelly and Goldstein (Donnelly and Goldstein 1970). It is a common method for the isolation, fractionation, and purification of carbohydrate-containing structures (Mechref et al. 2008; Fanayan et al. 2012). For instance, concanavalin A (Con A) is the most widely used lectin and has been employed for glycoprotein/glycopeptide enrichment due to its affinity in identifying *N*-glycan high mannose core (Feng et al. 2009). Because of the low affinity between lectins and carbohydrates, glycans can be competitively displaced from the complex by a competitor compound. Also, due to the low specificity of some lectins, two or more glycans may bind to a carrier during affinity chromatography (Durham and Regnier 2006). On the other hand, other lectins have narrow specificity for unique glycan structures, such as L-phytohemagglutinin (L-PHA) for the targeted β-1,6-branched *N*-linked glycan enrichment (Ahn et al. 2010b) and jacalin for galactosyl-β-1,3-*N*-acetylgalactosamine or *O*-linked GalNAc core structures in certain glycosylated proteins (Roque-Barreira and Campos-Neto 1985).

Durham and Regnier used the combination of Con A and lectin from *Artocarpus altilis* to isolate *O*-glycosylated peptides in the study of *O*-glycosylation sites of human serum proteins (Durham and Regnier 2006). Totten et al. (2018) used lectin affinity chromatography to separate core fucosylated and highly branched protein glycoforms. Another group applied lectin affinity chromatography on a column with sepharose-immobilized *Artocarpus incisa* lectin to study the correlation between changes in protein glycosylation and the progression of breast cancer (Lobo et al. 2017). Lectin chromatography has also been used for the purification of IgA

glycoforms from sera of IgA nephropathy patients and healthy controls (Amore et al. 2001).

Due to its affinity for core 1 type *O*-glycans, a jackfruit (*Artocarpus integrifolia*) lectin jacalin has been used for binding and purification of *O*-glycosylated proteins such as IgA1 (Hortin and Trimpe 1990), and several animal IgGs that are suspected of carrying *O*-glycosylation, such as rabbit IgG (Kabir and Gerwig 1997) and bovine IgG (Porto et al. 2007). Moreover, it enables the separation of IgA1 from the IgA2 subclass. Binding to the jacalin agarose column does not require divalent cations and is not significantly affected by ionic strength and pH over a wide range. However, it was observed it has a lower affinity for disialylated and higher affinity to monosialylated and non-sialylated IgG3 *O*-glycans (Plomp et al. 2015). Moreover, it was shown that jacalin could also bind to mannose and galactose residues in *N*-glycans (Bourne et al. 2002), so it is advisable to remove *N*-glycans with PNGase F and/or exoglycosidases before *O*-glycopeptide enrichment (Bai et al. 2015).

S*ambucus nigra agglutinin* (SNA) lectin fractionation was used by Kaneko et al. to enrich intravenous immunoglobulin G (IVIg) for sialylated IgG and to determine the role of sialylation in its anti-inflammatory activity (Kaneko et al. 2006). IVIg is a therapeutic IgG preparation initially developed as a replacement agent for treating primary and secondary Ab deficiencies (Guhr et al. 2011) and was shown to be beneficial to some patients with acute and chronic autoimmune diseases (Kumar et al. 2006; Imbach 1991). Equivalent approach of IVIg enrichment for sialylated IgG by SNA lectin fractionation was used by Gurh et al. (2011), whose results indicated that this approach is not a suitable method to enrich IVIg specific for Fc-sialylated IgG, because the IVIg is predominantly enriched for Fab-sialylated IgG.

2.3.2 *Lectin Microarrays*

Lectin-based microarrays have been used to analyze glycan profiles of purified glycoproteins, as well as for the characterization of therapeutic glycoproteins, including mAb (Hirabayashi et al. 2013, 2015; Syed et al. 2016) (Fig. 2.2). In this technology, several lectins with known specificity are immobilized as microdots on a

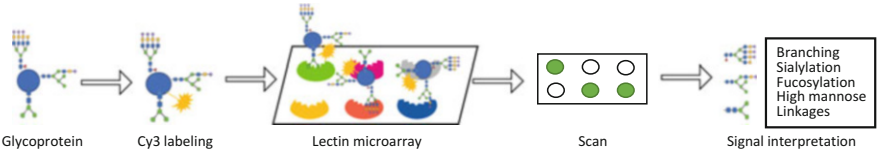

Fig. 2.2 Lectin-based microarrays have been used to analyze glycan profiles of purified glycoproteins. Glycoproteins are labeled with a fluorescent dye (Cy3) and then applied to the lectin chips. The binding signal at each lectin spot is measured, and the presence or absence of glycan variants in the testing sample detected based on the known selectivity of lectins toward glycan structures. Adapted from Zhang et al. (2016)

solid glass surface which is activated by chemical (e.g., epoxy, *N*-hydroxysuccinimide (NHS), amino, gold) or biochemical (e.g., streptavidin) derivatization procedures. After immobilization, residual activated groups are blocked (e.g., with amine or glycan-free serum albumin). Interaction of carbohydrate residues with the corresponding lectins can be detected either directly through their prior labeling with fluorescent reagents (e.g., Cy3 monoreactive dye) (Zhang et al. 2016) or indirectly—e.g., by overlaying a fluorescently labeled Ab (if available) against the target glycoprotein. After the extensive washing of the unbound probe, specific lectin-glycan interactions are detected with very high accuracy (Hirabayashi et al. 2013). To detect such interactions, confocal fluorescence and evanescent-field activated fluorescence can be used (Hirabayashi et al. 2013; Kuno et al. 2005). Methods such as bimolecular fluorescence quenching and recovery detection do not require the preliminary labeling of target carbohydrates (Koshi et al. 2006). Lectin microarrays are robust in generating glycan profiles that are generally consistent with the known glycan characteristics of an individual glycoprotein. They enable direct glycoprotein analysis, both as isolated glycoproteins or from body fluids (Rosenfeld et al. 2007), and are often used for rapid screening of Ig drugs.

Zhang et al. (2016), using commercial lectin chips, were able to determine glycan profiles of therapeutic mAbs and perform glycan profiling of proteins produced by different host cell systems. Furthermore, they evaluated the utility of lectin microarray in monitoring terminal galactosylation and sialylation of glycoproteins. Lectins that selectively bind core fucose, *N*-acetyl-D-lactosamine (Galβ1-4GlcNAc), mannose, sialic acid, or GlcNAc, which are structures commonly found in recombinant glycoproteins, were used (Hirabayashi et al. 2013).

Lectin microarray is sensitive to alterations in the terminal glycan structures, i.e., galactosylation vs. sialylation, and can effectively distinguish glycan isomers containing different sialic acid linkages (Zhang et al. 2016). Furthermore, such a platform demonstrates the usefulness of the lectin microarray in screening glycan patterns of protein samples and increased full coverage of all glycan variants of a glycoprotein. Despite all advantages, assay performance could be improved using lectins with advanced selectivity and binding affinity to distinct glycan species. One of the advantages is the possibility of lectin microarray customization by including lectins relevant to the glycan species that are possibly present in the testing sample. Lectin microarrays are commercially available by different producers.

Nowadays, carbohydrate-binding proteins like lectins play an important role in the structural and functional elucidation of glycoproteins, as well as in the study of their binding affinities and interactions with other proteins (Roucka et al. 2017). In contrast to Abs, lectins are generally more stable, more affordable, better characterized and address a broader spectrum of glycoproteins. As mentioned before, MS, UHPLC, and CE are the most common methods for Ig glycan analysis. While MS analysis mostly involves enzymatic digestion of a glycoprotein, UHPLC and CE usually require the release of glycans from a glycoprotein. In contrast, lectin microarrays enable rapid and direct measurement of glycan profiles on an intact protein (including *O*-glycans) without the need for protein digestion and glycan release. Therefore, the lectin microarray platform could be adopted as a

complementary tool for the high-throughput screening of glycan profiles of therapeutic glycoproteins.

2.4 Perspectives

In the last decade, the glycobiology field has significantly advanced in terms of the analytical capabilities of available technologies that facilitated our understanding of the various biological roles of Igs (and other glycoproteins). A correlation between changes in Ig *N*- and *O*-glycan moieties and the pathogenesis of many diseases, including inflammatory diseases, immune deficiencies, cardiovascular diseases, and cancer, has fostered a continuing interest in the elucidation of their functions.

Thus, the development of new and optimization of existing high-throughput approaches is crucial. Most lectin- and LC-based glycan sample preparation workflows developed to date are impacted considerably by the increase in robustness, throughput, sensitivity, integration of different analytical strategies, and hyphenation of orthogonal technologies (e.g., LC and MS).

Regarding the analytical techniques, the requirement for rapid and more detailed characterization of Ig glycosylation on all levels (free glycans, glycopeptides, subunit level, and intact Ig level) will lead to an increase in the application of lectin- and LC-based methods combined with mass spectrometric techniques. Development of fast and robust purification and enrichment techniques that would enable low-abundant Igs isolation and their in-depth glycan analysis remains a challenge. Lectin microarrays could prove to be useful here since they do not require additional steps of protein digestion or glycan cleaving that usually lead to sample losses and make glycan analysis of low-abundant Igs even more demanding.

Employment of rapid, sensitive protocols enabling Ig glycosylation analysis from low amounts of starting material already allows glycan detection and quantification using multiple analytical technologies without the necessity for separate sample preparation, significantly decreasing hands-on time. Due to the higher degree of automation and throughput increase, separation techniques coupled with mass spectrometric detection will dominate the field. With the development of automated, coupled techniques, the development of bioinformatics tools is essential.

To summarize, the analytical techniques applied for Igs glycosylation analysis will further develop toward a more detailed and sensitive characterization of *N*- and *O*-glycosylation but also toward fast, automated high-throughput methods for glycosylation monitoring.

Compliance with Ethical Standards

Conflict of Interest T. Petrović and I. Trbojević-Akmačić are employees of Genos Ltd., a private research organization that specializes in high-throughput glycomic analysis.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 3
Mass Spectrometry-Based Methods for Immunoglobulin G *N*-Glycosylation Analysis

Siniša Habazin, Jerko Štambuk, Jelena Šimunović, Toma Keser, Genadij Razdorov, and Mislav Novokmet

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S. Habazin · J. Štambuk · J. Šimunović · G. Razdorov · M. Novokmet (✉)
Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia
e-mail: mnovokmet@genos.hr

T. Keser
Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

M. Pezer (ed.), *Antibody Glycosylation*, Experientia Supplementum 112,
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Abstract Mass spectrometry and its hyphenated techniques enabled by the improvements in liquid chromatography, capillary electrophoresis, novel ionization, and fragmentation modes are truly a cornerstone of robust and reliable protein glycosylation analysis. Boost in immunoglobulin G (IgG) glycan and glycopeptide profiling demands for both applied biomedical and research applications has brought many new advances in the field in terms of technical innovations, sample preparation, improved throughput, and confidence in glycan structural characterization. This chapter summarizes mass spectrometry basics, focusing on IgG and monoclonal antibody *N*-glycosylation analysis on several complexity levels. Different approaches, including antibody enrichment, glycan release, labeling, and glycopeptide preparation and purification, are covered and illustrated with recent breakthroughs and examples from the literature omitting excessive theoretical frameworks. Finally, selected highly popular methodologies in IgG glycoanalytics such as liquid chromatography–mass spectrometry and matrix-assisted laser desorption ionization are discussed more thoroughly yet in simple terms making this text a practical starting point either for the beginner in the field or an experienced clinician trying to make sense out of the IgG glycomic or glycoproteomic dataset.

Keywords Mass spectrometry · Liquid chromatography · Solid-phase extraction · Ion fragmentation · Glycomics · Glycoproteomics · Biomarker discovery · Antibodies · Immunoglobulins

Abbreviations

2-AA	2-aminobenzoic acid (anthranilic acid)
2-AB	2-aminobenzamide (anthranilamide)
2-AP	2-aminopyridine
4-HCCA	α-cyano-4-hydroxycinnamic acid
Abs	Antibodies
ACN	Acetonitrile
ANTS	8-aminonaphthalene-1,3,6-trisulfonate
APS-PEG	Aminopropylsilane-polyethylene glycol
APTS	8-aminopyrene-1,3,6-trisulfonate
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE–LIF	Capillary electrophoresis with laser-induced fluorescence detection
CE–MS	Capillary electrophoresis coupled to mass spectrometry
CFG	Consortium for Functional Glycomics
CGE	Capillary gel electrophoresis
CID	Collision-induced dissociation
CIEF	Capillary isoelectric focusing
Cl-CCA	4-chloro-α-cyanocinnamic acid
CNBr	Cyanogen bromide

CZE	Capillary zone electrophoresis
DHB	2,5-dihydroxybenzoic acid (gentisic acid)
DTT	Dithiothreitol
EACA	ε-aminocaproic acid
ECD	Electron capture dissociation
EDC	1-ethyl-3(−3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
ESI–MS	Electrospray ionization mass spectrometry
ETD	Electron transfer dissociation
EThcD	Electron transfer/higher energy collision dissociation
Fab	Fragment antigen-binding
FASP	Filter-aided sample preparation
Fc	Fragment crystallizable
Fmoc-Cl	Fluorenylmethoxycarbonyl chloride
FT-ICR	Fourier-transform ion cyclotron resonance
GlcNAc	*N*-acetylglucosamine
HCD	Higher-energy collision dissociation
HILIC	Hydrophilic interaction liquid chromatography
HILIC–SPE	Solid-phase extraction using hydrophilic interaction liquid chromatography
HILIC–UPLC–FLR	Hydrophilic interaction ultraperformance liquid chromatography with fluorescence detection
HOBt	1-hydroxybenzotriazole
HPAEC	High-pH anion exchange chromatography
IAA	Iodoacetamide
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
IM-MS	Ion mobility-mass spectrometry
ISD	In-source decay
ITP	Isotachophoresis
K_a	Equilibrium association constant
LC–MS	Liquid chromatography coupled with mass spectrometry
LDI	Laser desorption ionization
m/*z*	Mass-to-charge ratio
mAbs	Monoclonal antibodies
MALDI	Matrix-assisted laser desorption/ionization
MALDI–MS	MALDI coupled with mass spectrometry
MALDI-TOF–MS	MALDI coupled with time-of-flight mass spectrometry
MECC/MEKC	Micellar electrokinetic capillary chromatography
MEEKC	Microemulsion electrokinetic chromatography
MRM	Multiple reaction monitoring

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NACE	Nonaqueous capillary electrophoresis
nano-LC	Nano-liquid chromatography
Neu5Gc	*N*-glycolylneuraminic acid
NP-HPLC	Normal-phase high-performance liquid chromatography
pAbs	Polyclonal antibodies
PGC	Porous graphitized carbon
PGC-LC	Porous graphitized carbon liquid chromatography
PMP	1-phenyl-3-methyl-5-pyrazolone
PNGase F	Peptide:*N*-glycosidase F
PpL	Protein L
ProA	Procainamide
PTM	Posttranslational modification
Q-TOF	Quadrupole time-of-flight
RP	Reversed-phase
RP-LC	Reversed-phase liquid chromatography
SpA	Protein A
SpG	Protein G
TETA	Triethylenetetramine
TFA	Trifluoroacetic acid
TMT	Tandem mass tag
TOF	Time-of-flight
UPLC®	Ultra performance liquid chromatography
(U)HPLC–FLR–MS	(ultra)high-performance liquid chromatography with fluorescence detection coupled to mass spectrometry
ZIC-HILIC	Zwitterionic hydrophilic liquid interaction chromatography

3.1 Basic Principles of Mass Spectrometry

Since the beginning of the twentieth century, it is possible to manipulate individual gas-phase ions in a vacuum using electric and magnetic fields (Griffiths 2008). The study of matter that relies on individual ion gas-phase manipulation is called *mass spectrometry* (MS) (Gross 2004). Central to MS is the ability to separate gas-phase ions based on their mass and charge. The mass spectrometer is an instrument by which matter is: (1) *ionized* into gas-phase, (2) *manipulated and separated* on the level of individual ions, and eventually (3) *detected*. There are several techniques for all three phases of MS, with specific strengths and weaknesses, resulting in conceptually different instruments applicable to various analytical problems.

Results of the MS measurement are usually presented as a mass spectrum. The mass spectrum is a graph showing the intensity (proportional to the strength of detected signal) versus *m/z* (ratio of mass to charge) relationship. Intensity is always scaled relatively, often normalized to the base peak (the most intense peak) in the

mass spectrum. The *m/z* ratio is a dimensionless quantity defined as the ratio between an ion's mass and the unified atomic mass unit, divided by its charge number (regardless of the sign). To determine mass from *m/z*, one has to be aware of the measured ion charge state. Since most chemical elements on Earth are, in fact, mixtures of isotopes, differing only by their number of neutrons and consequently by their mass, almost any molecule measured by MS is separated into several isotopologues called the isotopic envelope. The nominal mass difference between neighboring isotopologues is 1*u* (1 neutron), and consequently, the *m/z* difference between them is equal to 1/*z*. Due to its high sensitivity, specificity, speed, and almost universal applicability, MS is a central analytical technique in many *omics* fields, including glycomics and proteomics (Aebersold and Mann 2003; Ruhaak et al. 2018).

3.1.1 Ionization

To study matter by MS, first, molecules have to be ionized into a gas-phase. Several techniques can do this. At the beginning of the 1990s, the first soft ionization techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) were invented (Tanaka et al. 1988; Fenn et al. 1989). These ionization techniques enabled MS of the large and fragile biomolecules, such as proteins, glycans, and glycoconjugates (Zaia 2010).

The ESI process involves a conductive spray needle under electrical potential containing molecules of interest in a solution (Smith et al. 1990). At the tip of the ESI needle, a Taylor cone is formed out of the liquid meniscus under the influence of an electric field gradient. A jet of highly charged droplets flows towards the counter electrode at atmospheric pressure. Charged droplets are further spliced into a plume of smaller ones, from which desolvated molecules of interest enter the gas-phase as adducts with small ions (proton, sodium cation, etc.). Adducts can contain single or multiple small ions, which define the charge state of the molecule of interest.

MALDI involves the co-crystallization of molecules of interest with matrix molecules from a solvent, usually in an array of spots on a metal plate (Karas and Krüger 2003). Crystals are irradiated with a laser beam. The matrix absorbs the energy from the laser beam, which promotes desorption into the gas-phase and ionization of the molecules of interest by the formation of adducts with small ions. As opposed to ESI, MALDI produces almost exclusively singly charged ions.

3.1.2 Gas-Phase Separation and Detection

Ions of interest enter the low-vacuum ion transfer region individually and are ready for the next phases of MS, which take place in the quadrupoles, ion traps, or collision cells supported with an array of ion optics for electrostatic manipulation of charged

particles. Individual gas-phase ions formed are subjected to Lorentz force if immersed in an electromagnetic field (Maher et al. 2015):

$$\vec{F} = q\left(\vec{E} + \vec{v} \times \vec{B}\right) \tag{3.1}$$

where F represents force, q charge, E electric field, v speed, and B magnetic field.

Since force is proportional to mass (m) and acceleration (a), which is equal to time (t) derivative of speed (v) or second time derivative of the path (r),

$$\vec{F} = m\vec{a} = m\frac{d\vec{v}}{dt} = m\frac{d^2\vec{r}}{dt^2} \tag{3.2}$$

it is possible to describe ion motion in space and time by these classical equations:

$$\left(\frac{m}{q}\right)\frac{d^2\vec{r}}{dt^2} = \vec{E} + \frac{d\vec{r}}{dt} \times \vec{B} \tag{3.3}$$

The previous equation shows that the ion mass to charge ratio is a principal concept for the MS.

The separation of individual ions is done by a mass analyzer (Haag 2016). There are a number of mass analyzer types for ion separation. The main difference between them is the mass-resolving power, where mass resolution is defined as a mass to mass difference ratio (m represents m/z):

$$\frac{m}{\Delta m'} \tag{3.4}$$

Briefly, by convention, the mass resolution is defined as a measure of how well the two ions with close m/z can be distinguished. Only one isotopic composition then corresponds to each spectral peak. Mass accuracy, on the other hand, describes the difference between measured and theoretical m/z.

Mass resolution spans several orders of magnitude and ranges from 10^3 up to over 10^6. For an ion with the m/z of 1000, this means complete signal separation at m/z 1001 (resolution 10^3) and 1,000,001 (resolution 10^6). Inversely proportional to mass resolution, mass accuracy is essential for analyte identification based on accurate mass measurements.

Other performance parameters of different mass analyzers are m/z range (mostly limited to around 2000), speed (ranging from microseconds to seconds), duty cycle (or efficiency), and dynamic range (max-to-min signal ratio).

Low-resolving mass analyzers with a mass resolution of over 10^3 are transmission quadrupole (Q) and quadrupole ion trap (QIT), both belonging to the scanning type (Miller and Denton 1986; Nolting et al. 2019). To measure a broad range of m/z signals, Q needs to scan over the stability region. For a given m/z, a quadrupole operates within the stability region only when the applied radio frequency (RF) and

direct current (DC) voltages combination enable the ions to pass through. The efficiency of scanning mode is usually very low and can be expressed as single *m/z* over the total number of *m/z* in the range scanned (for example, 1/2000 for *m/z* range of 2000). Both of these are rarely used for analyte identification based on accurate mass measurements. The quadrupole is mostly used as a mass filter for ion selection based on *m/z*, while QIT is used for structural studies due to its ability to select and fragment ions at multiple levels (see Sect. 3.1.3 on tandem MS). These analyzers are very affordable, almost maintenance-free, and require only a modest vacuum relieving the need for complex mass spectrometer vacuum systems.

Next in line are time-of-flight (TOF) analyzers with a middle mass-resolving power ($>10^4$) (Boesl 2017). This resolution is usually enough for the charge state determination of multiply charged ions produced by ESI. Time-of-flight mass analyzers are the fastest (a microsecond scan time), with the broadest mass range ($m/z > 10^4$).

Kingdon trap (KT), commercially called Orbitrap™, performs measurements with a high-resolving power of over 10^5 (Nolting et al. 2019; Eliuk and Makarov 2015). It is predominately used in proteomics and other -omics studies, where analytes from complex mixtures are identified by accurate mass measurement.

However, to achieve the high mass resolution needed for this type of analysis, KT needs long scan times. Since the mass resolution is inversely proportional to scan time, a compromise has to be made between the duration of the scan and the required mass resolution.

The very same trade-off is imminent when using Fourier transform ion cyclotron resonance (FT-ICR), a mass analyzer with an ultra-high mass-resolving power (Nolting et al. 2019). With the resolution of over 10^6, it is possible to separate individual isotopologues with the same mass number (for example, ^{15}N, ^{33}S, ^{13}C, and ^{2}H for $A + 1$ mass number, where A is the atomic mass number) and unambiguously determine the molecular formula (Nikolaev et al. 2012). This analyzer tends to be expensive and complex for usage, making it less popular in the omics field.

Detection of ions in MS occurs by charge counting or by charge-induced image current measurement. Charge counting is performed by the electron multiplier. When a beam of measured ions emerges from a mass analyzer and strikes a detector, it causes a release of an electron from the surface layer. During the process called secondary emission, a single electron induces the emission of additional ones, resulting in the amplification of the input current. All MS analyzers, except for FT-ICR and KT, are coupled to this type of detector. FT-ICR and KT use the measurement of the image current, where ions flying between the detector plates induce an image current, which can easily be transformed from the time domain to the frequency domain by the Fourier transform algorithm. The calculated frequency is proportional to *m/z* (Gross 2004).

3.1.3 Tandem MS

Since molecular isomers have the same molecular formula and consequently the same mass, they cannot be separated by MS directly. However, by combining two mass analyzers with an ion activation technique in between, it is possible to select an ion of interest by the first mass analyzer, activate and fragment it, and measure the resulting fragments by the second mass analyzer. This is, in essence, tandem MS (MS/MS), which enables different isomer separation by their fragmentation pattern (de Hoffmann 1996).

Ions can be activated by different techniques, but only collision-induced dissociation (CID) is used in all tandem instruments. Ions are activated by collisions with inert gas, usually in a collision cell. A few collisions are required to fragment a single molecular bond, which translates into ion fragmentation at the least stable bonds—a peptide bond in peptides and a glycosidic bond in the case of the glycans (Wuhrer et al. 2007a). CID fragmentation mass spectrum contains a ladder of peptide fragments, usually single amino acid apart. Due to the linearity of the peptide's primary structure, this is usually enough to decipher the amino acid sequence. Annotation of glycan fragmentation mass spectrum is demanding because of branched glycan structures, and it is discussed in more detail later in the chapter.

Another popular activation method, at least for glycopeptides, is electron transfer dissociation (ETD) (Wuhrer et al. 2007a). It is based on the transfer of electrons from electron donor anions to analyte ions, which induces fragmentation of the glycosidic or peptide bonds (Syka et al. 2004; Kim and Pandey 2012).

Even though it is possible to combine any mass analyzers with the collision cell (q) in the tandem instrument, some combinations are more popular: triple quadrupole (QqQ), quadrupole time-of-flight (Q-TOF and Qq-TOF), quadrupole-Orbitrap (Q-KT), tandem time-of-flight (TOF-TOF) (Eliuk and Makarov 2015; Yost and Enke 1979; Chernushevich et al. 2001; Vestal and Campbell 2005). The transmission quadrupole (Q) is the ideal first mass analyzer due to its mass filtering capabilities.

There are two approaches to tandem experiments: *tandem-in-space* and *tandem-in-time* (de Hoffmann 1996). In tandem-in-space, ion isolation, activation, and fragment separation are being performed separately in space. On the other hand, the quadrupole ion trap instrument can only perform *tandem-in-time* experiments. By this approach, ions are isolated, activated, and fragments separated in the same space but sequentially in time. Another capability of the *tandem-in-time* instrument is the application of multiple levels of fragmentation or MS^n. The first stage of MS analysis is typically denoted as MS^1, and it involves only an ion selection process without any fragmentation. After the first level of fragmentation (MS^2), a single fragment can be isolated, activated, and fragmented in the second level of fragmentation (MS^3). The process can be performed in several cycles, achieving higher and higher fragmentation levels (e.g., MS^4, MS^5). This multilevel fragmentation can differentiate isomers with substructural differences. Quadrupole ion trap is the only analyzer capable of *tandem-in-time*, with up to 10 levels of fragmentation.

Each of the mass spectrometry solutions described in this introduction is applicable for antibody glycosylation analysis with certain advantages and disadvantages in throughput, user-friendliness, mass resolution and accuracy, ion fragmentation capabilities, and level of structural information obtained. In this way, ESI–MS(/MS) and MALDI–MS(/MS) systems are most commonly used for IgG glycoanalysis due to their moderate cost and broader availability. Both approaches are discussed more thoroughly in Sects. 3.5.1 and 3.5.2.

3.2 Levels of IgG *N*-Glycosylation Analysis

Immunoglobulin G has one conserved *N*-glycosylation site on each heavy chain in the fragment crystallizable (Fc) region at the Asn297. Additionally, only about 15–25% of the protein molecules have an *N*-glycosylation site at the IgG variable fragment antigen-binding (Fab) region (Holland et al. 2006; Stadlmann et al. 2010). The analysis of released IgG glycans (glycomics approach) gives an overall picture of *N*-glycome present on the protein, covering glycosylation of all IgG subclasses and both Fc and Fab fragments combined. Glycans still attached to the peptide backbone (glycopeptides) carry information about the site of glycosylation. Site-specific analysis reveals glycosylation site occupancy by different glycan structures. In the analysis of IgG glycopeptides (glycoproteomics approach), information about subclass-specific Fc glycosylation is obtained. However, insight on Fab glycosylation is missing, although it can still be analyzed on released glycan level. Site-specific glycosylation analysis of the Fab region of polyclonal IgG or IgG isolated from human serum is currently not viable. However, site-specific analysis of the Fc portion can give valuable information since its tryptic peptides enclose sequence differences depending on the IgG subclass (Selman et al. 2012a). Substantial heterogeneity, coming from both the constant and the variable region and the wide repertoire of possible glycan structures attached to it (Fig. 3.1) (Pučić et al. 2011), is masking the signals of possible Fab glycopeptides. Whatever approach is chosen, mass spectrometry-based techniques can help resolve glycan composition and its structural features.

Enzymatically released *N*-glycans can be analyzed in their unmodified form but are more often derivatized in different ways before the analysis. When analyzed in combination with separation-based methods such as liquid chromatography or capillary electrophoresis, glycans are primarily modified on their reducing end with one of the many different fluorescent dyes available (see Sect. 3.3.3). Other approaches include modification of hydroxyl and *N*-acetyl groups (permethylation), modification of sialic acid residues (esterification or methylation of carboxyl groups). Permethylation of released glycans also stabilizes sialic acids and enables efficient detection of a mixture of acidic and neutral glycans (Atwood et al. 2008; Kang et al. 2007). Esterification of sialic acids reveals the difference in the linkage types to underlying galactose residues (Fig. 3.1). Masses of esterified glycans will be

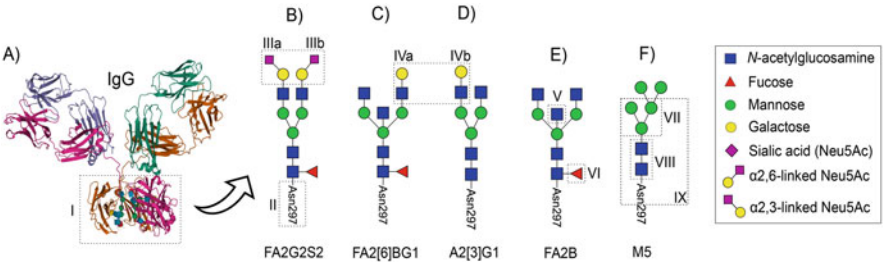


Fig. 3.1 The multi-layered IgG *N*-glycan diversity and structural motifs determine the choice of the MS analysis methods. A conserved IgG Fc region (**A**) *N*-glycosylation site (**I**) at Asn297 (**II**) is mainly occupied by biantennary glycans with structures spanning from disialylated (**B**), bisected (**C**, **E**), and monogalactosylated (**C**, **D**) to high-mannose (**F**). The sialic acid can be either α(2,3)- or α(2,6)-linked (**IIIa** and **IIIb**), while the antennary galactose residues can be present on 6-arm (**IVa**), 3-arm (**IVb**) or both in digalactosylated glycans (**B**). The bisecting GlcNAc (**V**) is β(1,4)-linked to the core mannose, while the core fucose (**VI**) is α(1,6)-linked to the innermost GlcNAc residue. About 90% of IgG glycans are core fucosylated. Minor glycan structures include, e.g., high-mannose structures (**F**). Further information on IgG glycosylation can be found in (Gudelj et al. 2018). All these glycans share some common features: the trimannosyl core (**VII**) and *N*,*N*′-diacetylchitobiose core (**VIII**), together with the asparagine residue from the peptide backbone, form the Man3GlcNAc2Asn *core* (**IX**). *For glycan depictions, rules proposed by the Consortium for Functional Glycomics* (*CFG*) *are followed. A textual IgG glycan nomenclature* (*below depictions* **B**–**F**) *can also be used:* F—α1,6-linked core fucose, A_n—number (*n*) of antennas (GlcNAcs) on a trimannosyl *N*-glycan core, G_n—number of β1,4-linked galactoses on 3- (Aebersold and Mann 2003) or 6-antenna (Fenn et al. 1989), B—bisecting GlcNAc, S_n—number of sialic acids (Neu5Ac) linked to galactoses, M_n—number (n) of mannoses

different if sialic acid residues are linked to galactose by α(2,6) versus α(2,3) linkage (Reiding et al. 2014).

Glycoproteomics is more challenging than traditional proteomics due to the high complexity and variability of glycan structures. Different MS-based protein and glycoprotein structural characterization approaches remain similar as the analysis can be performed on multiple structural levels. When an intact glycoprotein of interest is analyzed directly without fragmentation, the approach is called a native MS. Intact glycoprotein analysis gives information about its molecular weight but provides little structural insight. More recently, ion mobility-mass spectrometry (IM-MS) has seen considerable progress in new techniques for intact glycoprotein analysis, especially with the discovery of collision-induced protein unfolding in the gas-phase (Hernandez-Alba et al. 2018; Tian et al. 2015). Still, for structurally resolved information, the glycoprotein is usually cleaved into smaller fragments before or during the MS analysis. This fragmentation process can be performed either in the gas-phase inside a mass spectrometer or in-solution before the MS analysis by proteolytic cleavage. When the fragmentation happens primarily in the gas-phase, the mass spectrometer is used to analyze the entire molecule directly—this is called a "down"—type approach.

On the other hand, when the fragmentation happens primarily in solution, and the MS is used to analyze each fragment, the molecular structure is deduced on the

information obtained from the corresponding fragmentation spectra in an "up"-type approach.

The "up" and "down" methods are further divided into (1) top-down, (2) middle-up, (3) middle-down, and (4) bottom-up subtypes (Zhang et al. 2009). In a top-down method, structural information is obtained from the fragmentation pattern of the intact glycoprotein inside the MS. When the glycoprotein is cleaved into a few large fragments before being introduced into the MS, the approach involves a middle-up method if the masses of these fragments are determined directly or a middle-down method if further gas-phase ion fragmentation is performed. In a bottom-up method, a glycoprotein is first digested into smaller peptides and glycopeptides before being introduced into the MS, regardless of whether these peptides are fragmented during the analysis or not (Zhang et al. 2009; Lermyte et al. 2019). Glycoproteins can be analyzed at one additional level—MS of the released glycans. The glycan part can be enzymatically or chemically released from the protein part and then analyzed separately to explore the distribution and quantify and/or confirm the glycan structures by MS/MS. Both "up" and "down" approaches have their advantages and disadvantages in terms of structural resolution, glycosylation information, sequence coverage, sample consumption, cost, speed, and effort required for the analysis. Therefore, different techniques are often combined to provide an in-depth glycoprotein characterization (Ayoub et al. 2013; Tran et al. 2016). Figure 3.2 summarizes the

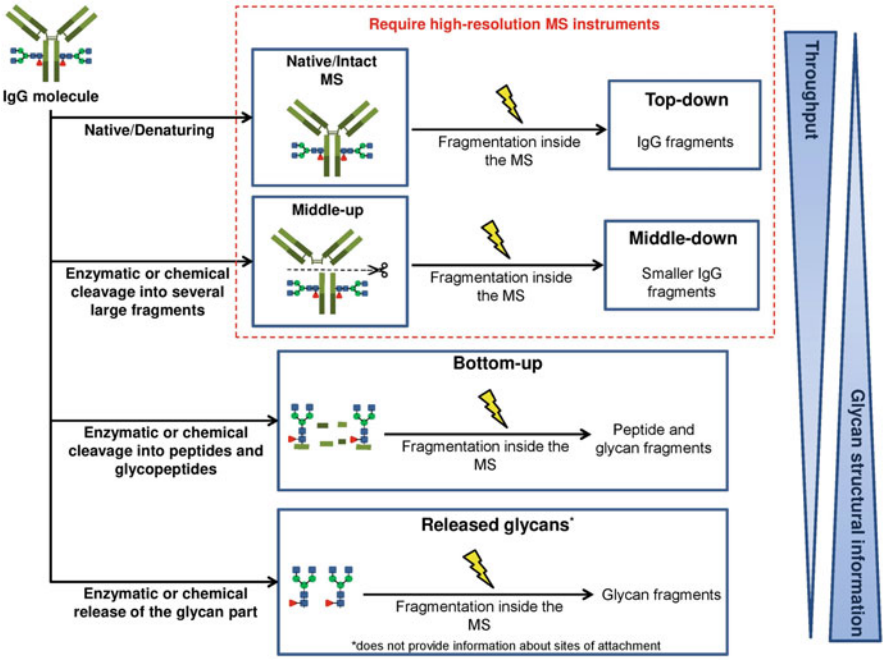


Fig. 3.2 Different levels of IgG structural characterization by MS-based methods

application of each of these approaches for the glycobiological structural characterization of immunoglobulins.

Analysis of IgG by a top-down approach consumes little sample, results in improved throughput, and can provide useful structural information in a very short time by reducing the number of sample preparation steps (Todoroki et al. 2020). The major drawback of a top-down MS for large proteins such as IgG is its limited structural resolution. The isotopic peaks of an intact IgG are usually not well resolved due to many different charge states. Therefore, the intact protein mass is difficult to determine, and high-resolution MS instruments are required. However, significant progress has been made recently in both top-down and native protein MS due to technological innovations, including improvements in ionization techniques and high-resolution detectors (Lermyte et al. 2019). Therefore, accurate MS analysis of intact antibodies is increasingly conducted in monoclonal antibodies (mAbs) production to rapidly detect major glycoforms (Tissot et al. 2009). The high-resolution instruments enable monitoring the degree of glycosylation of the intact molecule and the site-specific distribution of the major glycans, although the detection of minor glycoforms remains challenging (Zhang et al. 2009).

Middle-up IgG analysis involves cleaving the glycoprotein into several large fragments before the MS analysis. It provides a simple way to detect structural changes within a specific region of the molecule but cannot pinpoint the change to a specific amino acid residue (Zhang et al. 2009). Obviously, the site-specific information cannot be obtained this way. Yet, as each IgG subclass exists in several amino acid sequence variants (allotypes), it is possible to explore allotype-specific glycosylation IgG Fc glycosylation using the middle-up approach (Sénard et al. 2020). The IgG digestion can be achieved either chemically or by using structure-specific enzymes that are selective for only one or a handful of cleavage sites in the glycoprotein. For example, reduction of disulfide bridges can be employed to separate light and heavy IgG chains—this approach can be used either alone or in combination with proteolysis (Lermyte et al. 2019). On the other hand, bacterial IgG-degrading enzymes (see Sect. 3.3.4 and Fig. 3.3 for more details) (Ayoub et al. 2013) allow cleavage in the lower and upper hinge region, respectively (Lermyte et al. 2019). The high-resolution MS can provide accurate and often isotopically resolved mass spectra of these large fragments for further conclusions about both protein integrity and the presence of post-translational modifications, including glycosylation.

In the middle-down approach, as in the middle-up, chemical or enzymatic digestion is used to cleave the IgG into large fragments. However, instead of just measuring the mass of these fragments, they are also subsequently fragmented in the gas-phase during the MS analysis. This enables increased structural resolution with the benefit of simple sample preparation as separating the heavy and light chain prior to MS/MS analysis facilitates spectral assignment, while reduction of disulfide bridges allows for complete unfolding and improves dissociation efficiency (Lermyte et al. 2019). A middle-down proteomics approach using IdeS digestion has been used to assess the complexity, long-term storage stability, and biosimilarity of therapeutic antibody formulations (Todoroki et al. 2020).

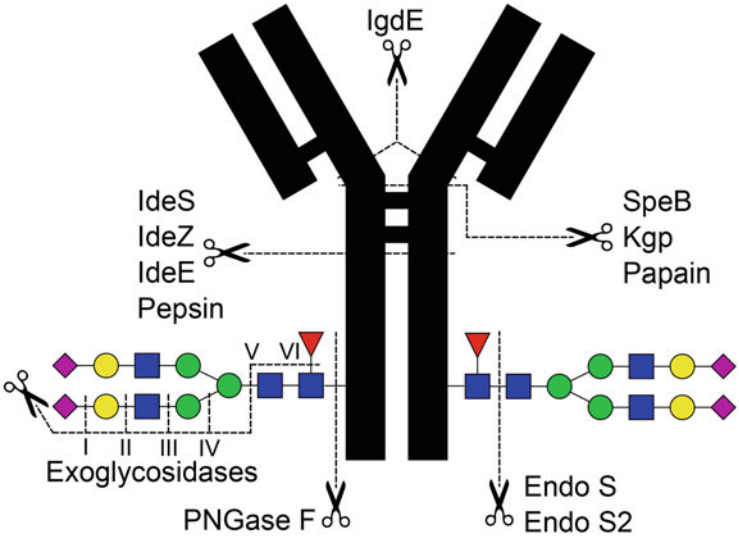


Fig. 3.3 Enzymology of IgG digestion strategies for glycoanalytics. Aside from endo- and exoglycosidases, different proteases are sometimes employed to fragment Ab molecules, drug-Ab conjugates, or Fc-fusion proteins for middle-up or middle-down glycoproteomics or to analyze Fc and Fab glycosylation separately (Sjögren et al. 2016). Bacterial proteases IdeS (von Pawel-Rammingen et al. 2002), IdeZ (Faid et al. 2018; Eriksson and Norgren 2003) and IdeE (Lannergård and Guss 2006) cleave IgG below hinge region, yielding $F(ab')_2$ and Fc fragments; similar to pepsin partially degrading Fc to pFc′. The IgdE (Spoerry et al. 2016) digests human IgG1 above the hinge and releases two Fab fragments (Faid et al. 2018). Streptococcal pyrogenic exotoxin B (SpeB) (Eriksson and Norgren 2003) and lysine-gingipain (Kgp) from *Porphyromonas gingivalis* (Vincents et al. 2011) are Cys-proteases cleaving in the hinge region above disulfide bridges. Papain similarly releases two Fab fragments from Fc, although when activated with 1–2 mM cysteine, $F(ab')_2$ can be obtained. Enzymes like neuraminidases (**I**), galactosidases (**II**), hexosaminidases (**III**), mannosidases (**IV**, **V**), and fucosidases (**VI**) of different linkage specificities are used for exoglycosidase glycan sequencing. Endoglycosidases cleave entire glycans either within the chitobiose core (Endo S, Endo S2) or between the innermost GlcNAc and Asn residue (PNGase F). Glycan depictions are for illustration purposes only and are unrelated to the enzymes' specificities

A bottom-up approach is the most frequently used for detailed structural characterization of IgG and its glycosylation as it can reveal the exact protein sequence and characterize glycosylation at the residue level simultaneously (Hinneburg et al. 2016). Contrary to the mass analysis of larger antibody fragments, the glycopeptide mass can be determined with much higher accuracy for greater confidence in the glycan composition assignment. In bottom-up methods, the IgG is cleaved into small peptides by a protease, followed by the MS or, more frequently MS/MS analysis. Bottom-up methods provide the most detailed structural information compared to the other approaches. However, on the downside, they are labor-intensive, lengthy, and often suffer from problems such as large sample consumption and artifacts introduced during digestion. Proteolytic IgG digestion is usually performed with trypsin or Lys-C after reduction and alkylation under denaturing conditions. Chemical

cleavage methods, such as cyanogen bromide (CNBr) digestion, are also used occasionally (Zhang et al. 2009). Bottom-up IgG glycosylation analysis provides information on the glycan structure, heterogeneity, and attachment sites (Ayoub et al. 2013). In cases where glycans are present at sites beyond the Fc region, such as *N*-glycosylation in the antibody Fab region, Fc glycoforms are typically analyzed at the glycopeptide level (Zhang et al. 2009).

IgG glycosylation profile can also be explored by releasing glycans from the protein part of the molecule. This approach enables complete coverage of glycan components, but it completely ignores the protein part and information about sites of attachment and site-specific glycan distribution. Glycans can be released from the antibody either chemically (e.g., hydrazinolysis) or enzymatically (e.g., peptide:N-glycosidase F or endoglycosidase F) (Zhang et al. 2009). Released glycans are usually chemically derivatized before the MS analysis in order to enhance the sensitivity of the analysis since free glycans lack chromophores or fluorophores and do not ionize well. Released glycans are most often analyzed with online fluorescence detection and MS/MS because it enables high sensitivity, relative quantitation, and structural information within a single chromatographic analysis (Zhang et al. 2009; Keser et al. 2018). Furthermore, by using antibody-based affinity capture and IgG-degrading enzymes (IdeS), it is also possible to obtain IgG Fab and Fc released glycan profiles separately (Bondt et al. 2014). Analysis of IgG glycosylation on a released glycan level is most quantitative and highly sensitive and can provide detailed structural information, but it needs to be combined with other approaches to include information about sites of attachment and site-specific glycan distribution.

3.3 Sample Preparation for IgG Glycosylation Analysis

3.3.1 Protein A, G, and L Affinity Chromatography for IgG Enrichment from Biological Samples

Fast, specific and simple enrichment of natural immunoglobulins (Ig) or engineered antibodies (Ab) is a prerequisite for streamlined glycomic and glycoproteomic high-throughput workflows. Affinity chromatography is typically used for enrichment. It utilizes reversible non-covalent—mainly electrostatic, van der Waals and hydrophobic—interactions between biomolecules and immobilized ligands. It facilitates the enrichment of, among others, monoclonal and polyclonal (pAbs) antibodies or their fragments from complex biological matrices (blood plasma and serum, ascites fluid, cell culture supernatants) for subsequent downstream characterization, additional purification, or their removal as potential contaminants. Samples are applied to chromatographic media, washed, and eluted under controlled conditions (pH, ionic strength, solvent polarity) that ensure optimal binding, minimal loss of the desired analyte(s) during the washing step and eventually high recovery without unwanted

Ab degradation (Moser and Hage 2010). Traditionally, purification of IgG and other immunoglobulins involved sequential precipitation with high concentrations of ammonium, sodium, or dextran sulfate (Lermyte et al. 2019; Ayoub et al. 2013). This approach is still used for large-scale production and, if followed by size-exclusion chromatography, offers a cost-effective alternative to affinity purification. IgG recovered in this way is contaminated with other co-precipitated proteins, which now makes affinity chromatography using recombinant bacterial protein A, G, or L as a ligand generally accepted and widely exploited in both preparative and analytical protocols. For convenience, protein ligands are typically immobilized to beaded matrices such as crosslinked agarose-derived Sepharose™, gold, or membrane surfaces using different bioconjugation chemistries (Faccio 2018). Increased availability and simplified synthesis of nanomaterials, polymeric substrates, and functionalized microparticles have also brought innovation to antibody affinity purification and immunoassays (Table 3.1).

3.3.1.1 Protein A

Protein A (SpA) is a 42 kDa surface protein produced by *Staphylococcus aureus,* possessing a high affinity (K_a 1.4×10^8 M^{-1}) (Choe et al. 2016) for the human IgG Fc region with a binding ratio of 1:2 (Yang et al. 2003). Its five binding domains (A–E) target the Ab between the constant heavy chain domains C_H2 and C_H3 (Choe et al. 2016).

Regarding human subclass specificity, protein A does not bind IgG_3 (except allotypes with His435 instead of Arg435) (Rispens and Vidarsson 2014), while it binds strongly to the rabbit, pig, and guinea pig IgG. Rat, chicken, and goat IgG do not bind to protein A at all (Page and Thorpe 2002). As with other immobilized bacterial proteins used for Ab purification, SpA requires low-pH for elution. This often leads to Ab denaturation or aggregation and can be detrimental to the protein's functional and structural properties, so alternative ways are sought through protein engineering to allow for milder elution conditions. One interesting approach was demonstrated by Koguma et al. by developing a thermoresponsive mutant of SpA that binds IgGs at low temperatures and releases them upon heating (Koguma et al. 2013). Another strategy involved the introduction of a calcium-dependent protein A-derived domain with high dynamic binding capacity, where complete elution was carried out at neutral pH (Kanje et al. 2018). A simple double-point mutation of His to Ser and Asn to Ala resulted in milder elution conditions compared to commercially available SpA, making it attractive to produce a novel affinity chromatography media with enhanced properties (Pabst et al. 2014). Other cost-effective options like mimetic SpA-like peptide ligands and peptidomimetics have also emerged (Faccio 2018; Choe et al. 2016).

Table 3.1 Selected recent examples of innovative protein-ligand immobilization strategies and applications for IgG enrichment and analysis

No.	Protein	Support	Sample(s)	Summary	References
1	A, AG	SiO_2 magnetic microspheres	Rabbit serum	Ligand immobilized to aldehyde-functionalized magnetic SiO_2 meso- and macroporous microbeads with the fast magnetic response, synthesized by hydrolysis-condensation approach. Reusable. Enriched IgG to >95% purity. Incubation time: 1 h at room temperature	Salimi et al. (2018)
2	AG	β-D-glucan microspheres	Mouse and rabbit serum	Carbodiimide-linked protein AG conjugate microparticles based on β-D-glucan. Enriched IgG to 92% purity. Suitable for the enrichment of fusion proteins by co-immunoprecipitation for protein-protein interaction studies	Yang et al. (2020)
3	A	PP fibers	CHO cell culture supernatant spiked with human IgG1	Polypropylene capillary-channeled polymer fibers with absorbed SpA, packed into a microbore column. Samples loaded in phosphate buffer pH 7.4 (0.1 mL min^{-1}) and eluted with 4.5 mM H_3PO_4 (1.0 mL min^{-1})	Trang and Marcus (2017)
4	A	Agarose beads	Human serum, IgG-spiked human saliva	Innovative micro-bead injection technique (μ-BIS) adapted for a lab-on-valve platform for reproducible packing of microcolumns between two optic fibers. Suitable for microscale quantification of IgG from biological matrices using HRP-labeled detection antibody	Ramos et al. (2019)
5	A	Au nanodots	IgG, human plasma	Photoluminescent gold nanodots prepared by 11-mercaptoundecanoic acid-mediated self-assembly of gold nanoparticles were conjugated with protein SpA for capture and luminescence IgG assay in human plasma. Results are comparable with ELISA	Shiang et al. (2011)

(continued)

Table 3.1 (continued)

No.	Protein	Support	Sample(s)	Summary	References
6	G	96-well plate wells	Anti-EGFR antibody	Nunc-Immuno™ MicroWell™ MaxiSorp™ 96-well plate with on-plate immobilized antibody-protein G complex crosslinked with bis (sulfosuccinimidyl)suberate and dimethyl pimelimidate. Potentially reusable chemically active surface for antigen binding-based immunoassays or extraction of antibody-specific proteins	Korodi et al. (2020)
7	A, G	Au nanoparticles	Human plasma	Citrate-capped gold nanoparticles conjugated with Cys-tagged protein A or G for the depletion of IgG from human plasma or cell culture supernatants	Liu et al. (2019a)
8	G	SiO_2 and carboxylated PS microparticles	–	In-depth optimization study on bead material, size, surface area, incubation time, ligand density, and conjugation chemistry influencing antibody binding and isolation efficiency	Ramirez et al. (2019)
9	G	PMMA monoliths	Human plasma	Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) epoxy-activated monolithic supports with immobilized protein G in the form of a 96-well filter plate. Reusable. Suitable for IgG enrichment in high-throughput glycomic and glycoproteomic workflows	Pučić et al. (2011)
10	L	PMMA monoliths	Rat serum	Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) epoxy-activated monolithic supports with immobilized recombinant protein L in the form of a 96-well filter plate. Reusable. Used for high-throughput IgG enrichment from Wistar rat serum	Habazin et al. (2019)

Abbreviations: *CHO* Chinese hamster ovary, *HRP* horseradish peroxidase, *EGFR* epidermal growth factor receptor, *PMMA* polymethacrylate, *PP* polypropylene, *PS* polystyrene

3.3.1.2 Protein G

Protein G (SpG) is another 30 kDa surface protein derived from group G *streptococci,* involved in bacterial pathogenesis (Fahnestock et al. 1986). It has three binding domains (C1–C3), and, unlike SpA, whose interaction with Ab Fc-domain is governed primarily by hydrophobic interactions, SpG exhibits pronounced hydrogen bonding and salt bridges as a dominant mode of binding of its C2 domain to the Fc (Yang et al. 2003; Rispens and Vidarsson 2014). Distinctive residues of Fc are also responsible for competitive binding to both SpA and SpG, which is not surprising since both proteins share a similar function on the surface of the bacterial cell wall (Sauer-Eriksson et al. 1995). SpG exhibits excellent binding capacity (K_a 6.7×10^9 M^{-1} towards human IgG) (Tran et al. 2016; Page and Thorpe 2002) for IgGs from a broad range of different species. All human and mouse IgG subclasses bind well to SpG (Sheng and Kong 2012). It is of note that wild-type SpG has an albumin-binding site, and for Ab purification and enrichment, a recombinant variant with suppressed albumin-binding capacity should be used to preclude co-enrichment of serum albumin (Choe et al. 2016).

3.3.1.3 Protein L

Multi-domain peptostreptococcal protein L (PpL) binds immunoglobulins via the interactions with the variable domain (V_L) of the κ-light chain (V_κ) rather than with the Fc-domain (Nilson et al. 1992). The affinity of PpL is not restricted to a particular immunoglobulin class but only to κ-light chain subgroups. The interaction is achieved through the two binding sites on each of five Ig-binding domains of PpL (B1-B5) and can be used to isolate various Ab fragments lacking Fc (Rodrigo et al. 2015). Hot spots for PpL interaction with the Ser-rich β-strand of V_κ are Tyr34 from site 1 and Tyr36 from site 2, as confirmed by X-ray crystallography (Svensson et al. 2004).

Protein L domains are capable of binding $V_{\kappa I}$, $V_{\kappa III}$, and $V_{\kappa IV}$ light chains of IgG (K_a 5.7×10^7 M^{-1}) (Choe et al. 2016) with no evident affinity for $V_{\kappa II}$ or λ subgroups, immunoglobulins heavy chains or constant domains (C_L) of a κ-light chain. The broad specificity and usefulness of this ligand have led to the development of engineered mutants capable of binding all κ-chains, with the potential to capture and purify any Ab fragment containing this light chain type (Lakhrif et al. 2016). Furthermore, by using salt additives in the elution buffers, the avidity of PpL can be fine-tuned to separate, e.g., high molecular weight Ab aggregates from monomers (Chen et al. 2020; Kihlberg et al. 1996; Nilsson et al. 1987).

3.3.1.4 Recombinant Fusion Proteins and Alternative Scaffolds

The Ig-binding domains of bacterial proteins can be combined into fusion proteins to expand binding properties (Choe et al. 2016). Thus, e.g., protein LG (Kihlberg et al. 1996), LA (Nilsson et al. 1987), and AG (Ghitescu et al. 1991) have been engineered and successfully employed as versatile novel ligands for Ab enrichment with broader specificity. Exhaustive reviews of less common immunoglobulin ligands were given by Mouratou et al. (2015) as well as Kruljec and Bratkovič (2017), covering natural and artificial proteins, aptamers, peptide ligands, and other small molecules used for Ab enrichment, depletion, and chromatography polishing steps.

3.3.2 Sample Preparation for Released IgG Glycan Analysis

Antibody *N*-glycosylation macro- and microheterogeneity manifest in the number of glycosylation sites and several glycan structural diversity levels (monosaccharide composition, size, branching, linkage, and charge). Two highly conserved Asn-297 glycosylation sites of the IgG heavy chains C_H2 domain are mostly occupied with covalently linked biantennary glycans (Fig. 3.1), affecting its stability, half-life, and role in the immune response, primarily through Fc-receptor (FcγR) interaction modulation (Gudelj et al. 2018). Together with the understudied and often neglected *N*-glycosylation within variable Fab domains (van de Bovenkamp et al. 2016), overall changes in IgG glycosylation profile such as increase or decrease in galactosylation, sialylation, bisection, and fucosylation reflect inflammatory status, biological age, and disease progression or regression of the individual. This makes contemporary multi-approach IgG glycoanalytics a valuable source of prospective biomarkers and renders it an indispensable tool in precision medicine and large-scale clinical cohort studies. IgG glycosylation analysis can be performed at different levels, targeting (1) intact glycoprotein, (2) cleaved Ab subunits, (3) released glycans, and (4) glycopeptides. The choice of the approach depends on the desired level of information, including Ab conformation, its subunit glycoforms, total released glycan diversity (glycome), glycan charge profile, and site-specific glycosylation microheterogeneity on glycopeptide level (see Sect. 3.2 and Fig. 3.2 for more details). Of the four aforementioned approaches, released IgG glycan and glycopeptide analyses are by far the most common as they mostly do not require complex state-of-the-art analytical instrumentation yet provide sufficient throughput, robustness, and versatility in glycobiomarker discovery, mAbs quality control, and diagnostic procedures.

Typically, all glycomic workflows include glycan release, labeling, enrichment, subsequent chromatographic or capillary electrophoresis separation coupled with mass spectrometry and/or fluorescence detection. All IgG *N*-glycans are of GlcNAcβ1-Asn type, assuming that innermost *N*-acetylglucosamine (GlcNAc) monosaccharide residue is attached to the polypeptide backbone asparagine (Asn)

within an Asn-*X*aa-Ser/Thr sequon, where *X*aa is any amino acid except proline (Stanley et al. 2017). The *N*-glycosidic bond between the Asn and the innermost GlcNAc can be hydrolyzed enzymatically using different endoglycosidases, commonly peptide-*N*-glycosidase F (PNGase F) (Huhn et al. 2009), by alkaline hydrolysis in the presence of hydroxylamine (Kameyama et al. 2018), or by oxidative release with sodium hypochlorite (Song et al. 2016). Released glycans are then enriched and purified either prior to or after labeling. Fluorescent and isotopic labels enhance glycan ionization and chromatographic behavior, facilitating their detection and separation. In this subsection, standard glycan release, labeling, and enrichment methods employed for IgG glycosylation profiling will be discussed and illustrated by recent advances.

3.3.2.1 Chemical and Enzymatic Glycan Release

A classical approach for chemical deglycosylation of both *N*- and *O*-glycans is hydrazinolysis (Kamerling and Gerwig 2007). It is a high-speed and reproducible option for IgG glycosylation analysis, outperforming enzymatic release with PNGase F (Kotsias et al. 2019). This reaction yields de-*N*-acetylated glycan hydrazone derivatives with simultaneous degradation of peptide backbone and formation of amino acid hydrazides. Upon hydrazine removal, free hydrazide amino groups are then re-*N*-acetylated using acetic anhydride, and the resulting β-acetohydrazide is acid-hydrolyzed to obtain free glycan with restored reducing terminus (Kamerling and Gerwig 2007). Since anhydrous hydrazine is a regulated and highly toxic substance, hydrazine monohydrate was tested as a safer replacement and shown to be almost equally efficient, even for large-scale preparations (Nakakita et al. 2007). Besides special hydrazine handling precautions, hydrazinolysis must be performed under strictly anhydrous conditions. The glycan "peeling" side-reaction reported for this approach can be precluded by the addition of ethylenediaminetetraacetic acid (EDTA) (Kozak et al. 2014) or malonic acid (Goso 2016). Of note, undesirable immunogenic *N*-glycolylneuraminic acid (Neu5Gc) residues on therapeutic mAbs glycans for human use are entirely degraded in this reaction, masking their presence (Kameyama et al. 2018). With the advent of new, fast-acting recombinant *N*- and *O*-glycosidases and enzyme-compatible detergents, hydrazinolysis tends to be outdated or replaced with safer modifications, especially for routine Ab glycosylation analyses.

Alternative chemical deglycosylation methods based on aqueous ammonia treatment with (Wang et al. 2018a) or without (Wang et al. 2018b) the presence of reducing agents, followed by derivatization with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) or Girard's reagent P, respectively, also exist. These workflows elegantly employ the formation of unstable glycosylamines and their subsequent conversion to the open-ring form of 1-amino alditols (Wang et al. 2018a) or simply back to glycans with reducing end termini upon ammonia removal (Wang et al. 2018b). Feasibility of these two new approaches was not directly demonstrated for IgG glycomics, although modified alkaline hydrolysis of monoclonal human IgG1

glycans with lithium hydroxide in the presence of hydroxylamine at 80 °C was published by Kameyama et al. Here, reducing glycans are further converted to rather more stable oximes and fluorescently labeled with 2-aminobenzamide (2-AB) tag, followed by 2-picoline borane reduction (Kameyama et al. 2018). The reaction causes some sialic acids and GlcNAc to deacylate even at lower temperatures, and the overall sialoglycan recovery was found to be half of that achieved with PNGase F. Care should be taken here if glycans with substantial quantities with sialic acids are to be analyzed.

Seeking a cost-effective protocol for producing large quantities of glycans from biological samples, the oxidative release of natural glycans (ORNG) using common household bleach was recently introduced (Song et al. 2016). Sodium hypochlorite (NaClO) used in ORNG degrades *N*- or *O*-glycans very slowly compared to peptides if reaction conditions are carefully controlled. The proposed mechanism involves chlorination of glycosylation site amide bond to give *N*-chloroamide, which is further converted to glycosylamines via glycan-isocyanate intermediates and finally hydrolyzed resulting in free reducing *N*-glycans. Bovine IgG and human plasma glycoproteins were successfully *N*-deglycosylated in this way, with results comparable to PNGase F treatment and a slightly better yield in fucosylated glycans, obviously more resistant to enzymatic release.

PNGase F from *Elizabethkingia meningoseptica* or *E. miricola* (Flavobacteriaceae) and peptide-*N*-glycosidase A (PNGase A) from almonds or rice are both asparagine amidases cleaving the linkage between the innermost GlcNAc and the Asn residue, converting it to aspartate (Wang and Voglmeir 2014) (Fig. 3.3). PNGase A can cleave *N*-glycans with or without α(1,3)-linked core fucose, while PNGase F can cleave only glycans without α(1,3) core fucosylation. PNGase F activity requires a minimal *N*,*N*′-diacetylchitobiose motif with the GlcNAc 2-acetamido group linked to Asn for recognition and is a widespread enzyme of choice for routine *N*-glycosylation analysis, of human IgG in particular (Table 3.2), as well as plasma or serum proteins (Huhn et al. 2009). A detailed investigation of PNGase F-mediated glycan release kinetics from human IgG tryptic glycopeptides (Huang and Orlando 2017) revealed that peptide backbone composition has a barely noticeable influence on the deglycosylation reaction rate. On the contrary, different glycan structures (fucosylated, bisected, mono-, and di-galactosylated, or sialylated) have been proven to influence and govern the PNGase F-catalyzed deglycosylation reaction rate. Thus, for example, core fucosylated glycoforms exhibit the fastest release rates while those sialylated and bisected are released slower. Following this, caution should be exercised and enzyme performance thoroughly tested when performing rapid IgG deglycosylation protocols of growing popularity (Cook et al. 2012). Novel bacterial *N*-glycosidases such as PNGase H^+ from *Terriglobus roseus* (Wang et al. 2014) with the combined activity of PNGase A and PNGase F or PNGase F-II, also from *E. meningoseptica* (Sun et al. 2015), with broader combined specificities towards glycans with α(1,3)- and/or α(1,6)-linked core fucose, were also described. The PNGase F is highly effective for complete and cost-effective glycan release in reducing and non-reducing conditions from different antibodies, regardless of their structure, and thus remains an enzyme of choice for IgG glycosylation analysis.

Table 3.2 Recent literature review on antibody PNGase F deglycosylation protocols and technical improvements

No.	Sample	Highlight	Summary	References
1	Commercial mAbs	Enzyme immobilized on SiO_2 beads and integrated into an LC chip	On-chip 6-s deglycosylation, glycan trapping, separation on C_8 stationary phase, and detection of released unlabeled glycosylamines. Enzyme microreactor, columns, and nano-ESI emitter tip were combined into a single microfluidic chip	Bynum et al. (2009)
2	Human IgG	Reusable magnetic beads with immobilized PNGase F	PNGase F-GST conjugated to glutathione-functionalized paramagnetic beads efficiently deglycosylated proteins in 10 min at 50 °C. Results are comparable with an in-solution reaction	Bodnar et al. (2016)
3		Comparison of different PNGases F for high-throughput glycomics	A detailed reproducibility and efficiency testing of enzymes from different producers for IgG and human plasma UPLC-FLR glycan analysis was performed.	Vilaj et al. (2020)
4		Rapid deglycosylation in a domestic microwave oven	A 20 min irradiation at 20% oven maximum power effectively catalyzed IgG enzymatic deglycosylation without notable loss of labile sialic acids	Zhou et al. (2012)
5		Integrated platform for online deglycosylation, HILIC enrichment, and labeling	An elaborate fluidic platform constructed from two 10-port valves for automated HILIC glycopeptide enrichment, N_2-assisted buffer exchange, hIMER deglycosylation, and C_{18} solid phase-assisted heavy/light dimethyl labeling is described	Weng et al. (2014)
6		PCT for rapid PNGase F deglycosylation	PCT device alternates cyclically between atmospheric and elevated pressure and enhances enzyme conformational changes and digestion site accessibility. IgG glycans were successfully released in 5 min with 1:2500 enzyme:Substrate molar ratio at 30 kPsi. Up to 12 samples can be processed simultaneously	Szabo et al. (2010)
7		PNGase F-GST immobilized on glutathione affinity resin	Model glycoproteins can be deglycosylated in 10–20 min at 50 °C by continuous aspiration/dispensing cycles using an automated protein purification platform	Szigeti et al. (2016)
8	–	Overview of different *N*-glycosidase immobilization approaches	A critical review of enzyme immobilization strategies (entrapment, aggregation, adsorption, covalent binding, microencapsulation), matrices, and substrates	Karav et al. (2017)

Abbreviations: *PCT* pressure-cycling technology, *F-GST* glutathione *S*-transferase, *hIMER* hydrophilic PNGase F immobilized enzymatic reactor, *nano-ESI* nano-electrospray ionization, *UPLC–FLR* ultraperformance liquid chromatography with fluorescence detection

Another attractive endoglycosidase for IgG glycosylation analysis is endoglycosidase S (Endo S) (Collin and Olsén 2001; Trastoy et al. 2018). This endo-β-*N*-acetylglucosaminidase from *Streptococcus pyogenes* hydrolyzes the β(1,4) linkage within the chitobiose core of complex glycans (Trastoy et al. 2018) and leaves the innermost GlcNAc residue—regardless of core fucosylation—attached to the peptide backbone (Fig. 3.3).

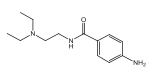
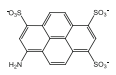
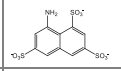
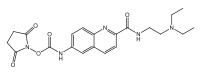
The structural background of Endo S interaction with different oligosaccharide moieties was recently studied in detail, applying X-ray crystallography, small-angle X-ray scattering, and molecular docking simulations (Trastoy et al. 2018). This enzyme was also proven to be useful for therapeutic mAb glycosylation engineering. In a study by Tong et al., transglycosylation of rituximab with glycan oxazolines upon Endo S digestion was performed, paving the way for more efficient antibody chemoenzymatic remodeling (Tong et al. 2018). A closely related *S. pyogenes* serotype M49 enzyme is Endo S2 (Sjögren et al. 2013), exhibiting pronounced specificity towards high-mannose and hybrid IgG glycoforms (Sjögren et al. 2015).

Overviews of sequential exoglycosidase digestion for antibody glycan analysis using capillary electrophoresis or liquid chromatography are given elsewhere (Sjögren et al. 2016; von Pawel-Rammingen et al. 2002), and this topic will not be discussed here in detail. An array of these glycoside hydrolases exist, e.g., α-neuraminidase, α-fucosidase, β-galactosidase, α-mannosidase, β-mannosidase, β-*N*-acetylglucosaminidase; with different linkage specificities (Fig. 3.3). The course of the sequencing reaction is usually monitored through the chromatographic retention time shift upon monosaccharide residue cleavage or using MS (see Sects. 3.4.5 and 3.5.1.2).

3.3.3 Fluorescent and Isotopic Glycan Labeling

Glycans are bulky hydrophilic biomolecules of low ionization efficiency, lacking chromophores. Numerous glycan derivatization strategies alleviate a bottleneck in detecting and quantifying these protein post-translational modifications (PTM) products. Typically, released glycan analysis uses a fluorogenic small-molecule reducing end tagging via (1) reductive amination of imines (Schiff bases) (Bigge et al. 1995), (2) Michael addition (Ruhaak et al. 2010a), (3) hydrazone, and (4) oxime formation with hydrazide or aminooxy reagents. Immunoglobulin G glycomic workflows still favor simple reductive amination labeling due to the low cost and high stability of secondary amines obtained. The reaction is typically performed in dimethyl sulfoxide in the presence of acetic acid and sodium cyanoborohydride ($NaBH_3CN$) as a reducing agent, overnight at 37 °C or 2–4 h at 60–65 °C. At higher temperatures, acid hydrolysis and loss of labile sialic acids can easily occur. Less toxic reducing agents, e.g., 2-methylpyridine (picoline) borane complex, are now of widespread use in IgG glycan labeling (Ruhaak et al. 2010b). Most of the labels used are aniline-, pyridine-, naphthalene-, or pyrene-derived (Table 3.3) and can be either neutral or negatively charged for charge-based

Table 3.3 Common fluorescent labels used in IgG released glycan analysis.

Label	Structure	Charge	Separation	Comments	References
2-aminobenzoic acid (2-AA)		(−)	LC, CE	Negatively charged label. It can also be used for negative mode MALDI-TOF–MS.	Jeong et al. (2018)
2-aminobenzamide (2-AB)		0	LC	Most widely used fluorescent label for IgG glycosylation analysis with FLR and/or MS detection.	Keser et al. (2018)
2-aminopyridine (2-AP)		0	LC	Used for separation and MS detection of isomeric IgG glycans on zwitterionic HILIC column.	Gong et al. (2013) and Albrecht et al. (2017)
Procainamide (ProA, DEAEAB)		0	LC	Protonation of the tertiary amine group greatly enhances signal intensities in positive mode ESI–MS. highly suitable for MS/MS structural glycan analysis.	Harvey (2000)
8-aminopyrene-1,3,6-trisulfonate (APTS)		(−)	LC, CE	Negative mode ESI–MS detection is also possible after HILIC–UPLC separation.	Reusch et al. (2014)
8-aminonaphthalene-1,3,6-trisulfonate (ANTS)		(−)	LC, CE	RP separation of ANTS-labeled glycans can also be achieved with the addition of ion-pairing reagents.	Takegawa et al. (2006) and Harvey (2000)
GlycoWorks™ *Rapi*Fluor-MS™		0	LC	NHS-carbamate dye. Labeling achieved in 5 min after digestion with rapid™ PNGase F. enhanced fluorescence and ionization properties. Available commercially only in the form of a kit. Higher MS sensitivity than 2-AB and ProA.	Tian et al. (2015) and Karav et al. (2017)

Abbreviations: *CE* capillary electrophoresis, *DEAEAB* *N*-(2-diethylamino)ethyl-4-aminobenzamide, *LC* liquid chromatography, *HILIC–UPLC* hydrophilic interaction ultraperformance liquid chromatography, *ESI–MS* electrospray ionization mass spectrometry, *FLR* fluorescence detection, *MALDI–TOF–MS* matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry, *NHS* *N*-hydroxysuccinimide, *RP* reversed-phase

separations. A new generation of fluorescent glycan labels is predominantly based on the *N*-hydroxysuccinimidyl (NHS) carbamate reactive group for fast labeling of *N*-glycosylamines (Lauber et al. 2015). Therefore, 'instant' 2-AB and ProA NHS-ester analogs have been synthesized (Vainauskas et al. 2018) as well as a very popular quinoline-based *GlycoWorks*™ *Rapi*Fluor-MS™ reagent (Waters Corporation, Milford, MA).

Modern proteomics heavily relies on stable heavy isotope (^{2}H, ^{18}O, ^{13}C, ^{15}N) labeling tags as such approach allows for multiplexing, i.e., simultaneous MS analysis of combined complex samples with enhanced ionization properties and accurate quantification by high-resolution MS (Gevaert et al. 2008). Among many variations, the most popular are isobaric tandem mass tags (TMT), based on the measurement of the ratio of diagnostic heavy and light reporter ion pairs upon TMT tag fragmentation (Thompson et al. 2003). Accordingly, glyco-TMTs were developed and used for mAb glycosylation analysis (Gong et al. 2013). Reductive amination duplex labeling with [$^{12}C_6$] and [$^{13}C_6$] aniline or 2-AA is also possible (Vainauskas et al. 2018; Gevaert et al. 2008). Internal standardization for MS-based glycomics has also been greatly improved by Zhou et al. with the development of the iGlycoMab (Zhou et al. 2016)—a murine mAb with integral ^{15}N-labeled glycans, which can be released, mixed with the sample, and analyzed to compensate for variations in sample preparation. For MALDI- and LC–MS, glycan permethylation with iodomethane (CH_3I) in the presence of sodium or potassium hydroxide is also used and is further described in Sects. 3.4 and 3.5.

3.3.4 Sample Preparation for Glycopeptide Mapping and Subclass-Specific IgG Fc Glycosylation Analysis

3.3.4.1 Enzymatic Digestion

Representative workflows that analyze site- and subclass-specific IgG glycosylation comprise in-gel or in-solution protease digestion of Ab, glycopeptide enrichment, chromatographic separation, and MS detection. Trypsin is typically used as a digestion enzyme, although protein conformation and glycoforms themselves can bias its activity. Falck et al. have pointed out that there is a digestion preference of trypsin for high mannose, bisected, and α(2,3)-sialylated IgG glycoforms over the abundant core fucosylated neutral ones and that it decreases when a chemical denaturation step is introduced before digestion (Falck et al. 2015). For high-throughput glycoproteomics, the denaturation step can be omitted for convenience. If needed, simple formic acid denaturation is sufficient (Falck et al. 2015), while MS-compatible RapiGest™ SF surfactant or traditional dithiothreitol (DTT) and iodoacetamide (IAA) reduction and alkylation can also be used (Yan et al. 2012). Microwave-assisted rapid trypsin digestion is an attractive alternative for mAb tryptic digestion, yet it can cause Asn deamidation artifacts (Formolo et al. 2014). Limited sequence coverage, multidimensional separations, and peptide fractionation

requirements in bottom-up mAb reductionist (glyco)proteomics and PTMs identification can be bypassed with the middle-up approaches made possible by an array of Ab proteases (Fig. 3.3). Allotype-specific HILIC– and CE–MS analysis of IgG glycoforms after on-beads IdeS digestion and isolation of Fc/2 subunits was achieved in this manner (Sénard et al. 2020). An excellent overview of alternative endoproteases such as Lys-C, Lys-N, Glu-C, or Asp-N is given by Tsiatsiani and Giansanti and in references therein (Giansanti et al. 2016; Kurogochi and Amano 2014). The different cleavage specificity of these enzymes can occasionally be utilized in case of too large tryptic peptides.

3.3.4.2 Stable Isotope Glycopeptide Labeling

Analytical benefits arising out of the stable isotope tagging in glycomics (see Sect. 3.3.3) can be translated to therapeutic and natural IgG glycoproteomics. Human IgG and IgG1 glycoforms from plasma cell myeloma were quantified with high sensitivity by MALDI-TOF-MS using benzoic acid light (D_0) and heavy (D_5) *N*-succinimidyl esters (Kurogochi and Amano 2014), while a similar approach with amino group-reactive succinic anhydride and its $D_4{}^{13}C_4$ isotopologue on IgG glycopeptides was demonstrated (Pabst et al. 2016).

More elaborate approaches include synthetic, isotopically labeled Fc glycopeptides for standard addition quantitative analysis (Roy et al. 2018) or SILu™MAb *s*table *i*sotope-*l*abeled *u*niversal *m*onoclonal *a*nti*b*ody for internal standardization combined with on-bead digestion and multiple reaction monitoring (MRM) mode MS analysis (Shiao et al. 2020).

Due to the high cost and limited commercial availability of these reagents, glycoproteomics still depends on intact unlabeled glycopeptide MS analysis and relative quantitation.

For glycosylation site occupancy analysis, PNGase F-mediated incorporation of ^{18}O into aspartic acid (+3 Da mass shift) upon digestion in $H_2{}^{18}O$ is a tool of the trade (Angel et al. 2007). Care should be taken here to avoid (or promote) residual trypsin variable incorporation of one or two ^{18}O atoms into peptides C-termini carboxyl groups. An interesting example of the MRM method developed to monitor serum IgG2, and IgA1 glycosylation site occupancy changes in the patients with congenital disorders of glycosylation and non-alcoholic fatty liver disease were developed by Hülsmeier et al. (2016).

3.3.4.3 Glycan and Glycopeptide Enrichment and Purification Strategies

As with other instrumental approaches, glycan or glycopeptide sample preparation is inseparable from technical advances in the LC-MS field. It destines the analytical workflow success, allows preset method validation criteria to be met, and further pushes the limits of detection, quantification, and structural characterization. The use

of different fluorescent or isotopic tags, low analyte concentrations, and presence of residual digestion enzymes or labeling reagents within a complex matrix of non-glycosylated peptides and proteins further complicate the choice of appropriate enrichment protocol. In their essence, all interaction modes target either the glycan or the peptide part via covalent (e.g., boronate and hydrazide chemistries) or non-covalent (e.g., HILIC, RP, ion exchange, lectins, PGC, TiO_2) interactions (Chen et al. 2014). The methods for both released IgG glycan and glycopeptide enrichment can frequently be used interchangeably as these analytes are of similar physicochemical properties.

A single exception is a reversed-phase chromatography, typically performed using C_8 or C_{18} functionalized silica, where hydrophobic interactions with the peptide moiety are utilized to desalt and enrich glycopeptides only, whereas released glycans cannot be purified in this manner. By tuning elution conditions, non-glycosylated peptides and contaminants are retained on or washed away from octyl- or octadecyl-modified silica. This enrichment mode is widely employed in high-throughput IgG glycopeptide enrichment for cohort studies due to low cost, speed, similar peptide hydrophobicities across subclasses, and low bias towards certain glycan types (Zaytseva et al. 2018; Alagesan et al. 2017).

Hydrophilic interaction liquid chromatography (HILIC) stationary phases and their zwitterionic modifications (ZIC®-HILIC sulfobetaine) (Alagesan et al. 2017) have seen the fastest expansion with dozens of innovative materials of broad or narrow affinity ranges and binding capacities developed.

An overview of the field, including discussion on retention mechanisms, recent breakthroughs, and future perspectives in HILIC-based materials for glycoanalytics, has been published recently (Chen et al. 2014; Selman et al. 2011). This form of normal-phase liquid chromatography has now been improved almost to a level of molecular recognition for specific carbohydrate structures. It can be technically adapted for comprehensive non-targeted protein glycosylation analysis or high-throughput screening in the forms of high-performance liquid chromatography, filter-aided sample preparation (FASP), 96-well filter plates, and pipette tip-based devices.

A lineup of new adsorbents and (nano)composites proved to be a successful alternative to the traditional IgG and mAbs glycopeptide purification approaches. Here, of particular interest are environmentally friendly (ligno)cellulosic materials, including cellulose microspheres (Sha et al. 2018), unmodified (Selman et al. 2011), and boric acid functionalized TiO_2-modified (Liu et al. 2019b) cotton wool, delignified balsa wood (Zhou et al. 2020), and boronic acid@fibrous cellulose (Sajid et al. 2020). Carrageenan on graphene oxide/poly(ethylenimine) support (Chen et al. 2019), freeze-casted honeycomb chitosan membrane (Zhang et al. 2019), Ti^{4+}-immobilized dendritic polyglycerol-coated chitosan (Zou et al. 2017), as well as carboxymethyl chitosan (Bodnar and Perreault 2015) are more elaborate examples of chemically remodeled linear polysaccharides with demonstrated excellent binding capacity, stability, and specificity for IgG glycopeptide enrichment.

Porous graphitic carbon (PGC)—a versatile stationary phase embracing several different binding interactions—is also effective for microscale purification and

chromatography of native and tagged glycans for biosimilars *N*-glycosylation profiling (Liu et al. 2017). Most enrichment modes can be conveniently combined for enhanced results. Ohta and coworkers have, accordingly, constructed a simple stop-and-go HILIC/RP-SPE tip (StageTip) for IgG1 glycopeptide capture (Ohta et al. 2017).

Surface engineering can greatly expand the chemical space of two-dimensional (2D) materials, and it has expectedly transformed sample preparation for protein glycosylation analysis. Several outstanding illustrations of new 2D material platforms for IgG glycoproteomics involve hydrophilic magnetic graphene coated with phytic acid for combined phospho- and glycopeptide enrichment (Hong et al. 2018), polydopamine-coated graphene decorated with magnetic Fe_3O_4 and chitosan (Bi et al. 2020), MoS_2/Au-nanoparticle-L-cysteine nanocomposite and, its variant with Au-nanowires grown on SiO_2 coating offering larger surface area for generous L-cysteine grafting and enhanced hydrophilicity. Unfortunately, novel techniques are typically limited to the labs where they were developed, and seldom are these materials and reagents widely available commercially.

For large-scale glycoanalysis, automation of robust and cost-effective glycan and glycopeptide enrichment is essential as it allows for the full diagnostic potential of protein glycosylation to be utilized. In this setting, glycoblotting for on-bead free reducing end glycan enrichment and fluorescent labeling seems to be promising (Amano and Nishimura 2010). Glycans are first ligated to hydrazide beads for either subsequent release and aminooxy-fluorophore tagging via *trans*-imination (BlotGlyco H®) or reduction of disulfide spacer after hydrazone bond formation with a predesignated fluorophore already coupled to a bead (BlotGlyco ABC®) (Miura et al. 2008). Sialic acids may also be on-bead methyl-esterified using 3-methyl-1-(*p*-tolyl)triazene for linkage-specific analysis if desired. A dedicated liquid handling platforms are fully compatible with BlotGlyco workflows (Nishimura 2011). Recently, glycoblotting was shown to fit the needs of large-scale human serum and IgG glycomics for cancer glycobiomarker discovery (Gebrehiwot et al. 2019).

Hydrophilic polypropylene 96-well filter plates are a straightforward option for HILIC purification of 2-AB- or ProA-tagged released glycans before (Ultra) High-performance Liquid Chromatography analysis coupled with mass spectrometry as they are compatible with vacuum manifolds and pipetting robots (Tian et al. 2015; Menni et al. 2013; Goh and Ng 2018).

3.4 Deciphering the IgG Glycan Structure

N-glycans found on IgG are oligomeric carbohydrate structures of different monosaccharides linked to each other by a glycosidic bond in a complex, branched manner. A glycosidic bond can be formed between an anomeric carbon of one monosaccharide and potentially any other hydroxyl group of the other sugar. Moreover, more than one hydroxyl group on any monosaccharide can be occupied

simultaneously, adding even more to the glycan complexity (Varki et al. 2015). When compared to linear biomolecules targeted by omics studies, such as DNA or proteins, glycans exhibit several orders of magnitude higher complexity and therefore require a more comprehensive approach to identify their compositional and structural features. There are several different methodological approaches to identify the structure of a glycan successfully. Here we will focus on the opportunities MS approaches are offering in this demanding endeavor.

Mass spectrometry is a powerful tool for glycan analysis, and in combination with several separation techniques (HPLC, nano-LC, UHPLC, CE) coupled to a mass analyzer, it can provide an almost complete picture of the analyzed sugar molecule. High-resolution instruments offer information about the glycan candidates already on the MS^1 level. Given the known information about the analyzed sample, such as biological type/origin, or biosynthetic pathways of analyzed glycans, even the *m/z* value alone can be very informative. In the case of IgG, a repertoire of possible constituent monosaccharides is very well known and defined by human biology, i.e., its sugar donors, biosynthetic pathway, glycosyltransferases, and glycosidases. Even more, IgG from human plasma has been characterized in detail (Pučić et al. 2011; Kobata 2008), which makes the elucidation of its glycan structures in further epidemiological and clinical studies rather simpler. Although this is true for the IgG from human plasma, the analysis of recombinant antibodies or biofluids with low IgG concentrations is not as straightforward. Glycosylation is a post-translational modification governed by a very complex system of enzymes, and it is dependent on a number of environmental factors (Menni et al. 2013). All these factors play an important role in the production of recombinant proteins in different cell lines under different conditions. Hence, IgG produced in different setups can yield a repertoire of possible glycan species attached to the consensus *N*-glycosylation sequence (Goh and Ng 2018). It is therefore important to use analytical techniques that can undoubtedly confirm the composition and structure of analyzed *N*-glycans.

3.4.1 Fragmentation

Even though a number of different analytical techniques are developed for the analysis of *N*-glycans, mass spectrometry-based techniques remain the most used analytical approach (Harvey 2020). As mentioned above, MS spectra obtained with high-resolution mass analyzers can provide good enough clues about the glycan identity. However, due to oligosaccharide complexity, a number of isobaric structures can have the same *m/z* value. A standard technique to overcome the issue of isomers in the characterization of glycan structures has become MS/MS (tandem MS or MS^n), where a single ion of interest can be selected (precursor ion) and later fragmented. Tandem MS becomes exceptionally informative in the ion trapping instrumental setup as it enables sequential fragmentation events. In the multiple stages of MS^n experiments, any fragment ion can be selected and subjected to

another round of fragmentation (Ashline et al. 2007). With the appropriate fragmentation method in the MS/MS experiment, each sugar molecule undergoes a series of linkage cleavages and shows a specific pattern of fragments. Numerous fragments obtained can be very informative about the original sugar composition and the type of linkages between constituent monosaccharides. Several tandem MS methods are developed and used for the analysis of oligosaccharides: collision-induced dissociation (CID), higher energy collisional dissociation (HCD), and electron-based dissociations: electron capture dissociation (ECD) and electron-transfer dissociation (ETD). CID is one of the first approaches that have been developed, and it is the most widely used method for the fragmentation of glycan structures (Sobott et al. 2009). During a CID event, the kinetic energy of the selected parent ion is increased as the ion enters a collision cell of the instrument. The ion collides with inert gas molecules within the cell, and the kinetic energy of the excited ion is transferred to vibrational energy, which results in bond cleavages (An and Lebrilla 2011). Collision gases commonly used in fragmentation experiments are nitrogen (N_2), helium (He), and argon (Ar). HCD is a form of CID, designed explicitly for Orbitrap mass analyzers (Jedrychowski et al. 2011). Levels of fragmentation energies are similar to CID. ECD is a fragmentation method introduced relatively recently (Zubarev et al. 1998). It is based on the irradiation of multiply charged ions with low-energy electrons. The electrons are captured by the irradiated ions resulting in dominantly c- and z-fragments in peptide fragmentation events. The c- and z-ion series are generated by the fragmentation of the C_α-N bond. The ETD shares the same mechanism of dissociation as ECD, with the main difference being the source of electrons. In ETD, the electron transfer occurs from singly charged reagent anions to multiply charged protonated peptides (Wuhrer et al. 2007a). A fluoranthene is the most common ETD reagent used.

3.4.2 Fragmentation Nomenclature

For the interpretation of generated fragmentation spectra, Domon and Costello proposed nomenclature of glycan fragments to simplify the communication of the often complicated MS/MS spectra (Domon and Costello 1988). The glycan fragmentation nomenclature is distinguished from the peptide by capital letters used in carbohydrate fragment spectra annotation. After a fragmentation event, ions can retain charge on either the reducing or non-reducing end of the oligosaccharide. Fragments with the charge at the reducing end are labeled X, Y, and Z, while ions with the charge at the non-reducing end are labeled A, B, and C. A- and X-ions are a result of a cross-ring fragmentation, while Y-, Z-, B-, and C-ions represent the cleavage of the glycosidic bond (Domon and Costello 1988). Subscript and superscript numbers are used to designate the exact location of glycosidic bond cleavage and the bond that has been cleaved following the cross-ring fragmentation, respectively, counting from the reducing end of the glycan chain. Figure 3.4 shows a

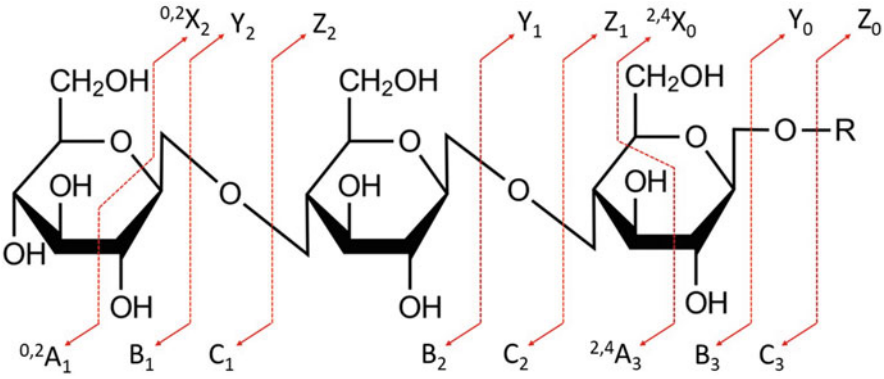


Fig. 3.4 Carbohydrate fragmentation nomenclature as proposed by Domon and Costello in 1988

schematic representation of nomenclature used in the interpretation of oligosaccharide fragmentation spectra introduced by Domon and Costello.

3.4.3 Fragmentation Candidates

Glycans can be fragmented either released or still attached to the peptide backbone asparagine. The choice of the approach is dependent on the information needed.

3.4.3.1 Fragmentation of Glycopeptides

Fragmentation of the tryptic glycopeptides can provide confirmation of both the peptide sequence and the glycan conjugate composition and its structural features. The CID experiments result dominantly in the cleavage of glycosidic bonds while the amide bonds of the peptide remain mostly intact (Conboy and Henion 1992; Huddleston et al. 1993). Fragmentation spectra are dominated by carbohydrate B- and Y-ions. Although CID favors glycosidic bond cleavage, information about peptide and glycan moiety can be obtained in a single MS/MS experiment by optimizing the collision energy stepping (Hinneburg et al. 2016). With the instrumental setup that allows multiple sequential fragmentation steps, tandem MS experiments can resolve both glycan and peptide sequences. After the MS^2 experiment, one of the most abundant fragments is a complete peptide with the first chitobiose core GlcNAc still attached to the *N*-glycosylation site. By choosing this fragment for further MS^3 experiments, we can obtain the amino acid sequence of the glycopeptide (Demelbauer et al. 2004). Another approach is to use complementary fragmentation events of ETD and HCD (EThcD). A diagnostic ion generated by neutral loss in the HCD event can be set to trigger sequential fragmentation of the parent ion by ETD. Both spectra are then recorded, with the HCD spectra dominated by B- and Y-ions

generated by cleavage of glycosidic bonds and the ETD spectra dominated by b- and y-ions generated by cleavage of amide bonds (Singh et al. 2012). However, the downside of ETD is the requirement of multiply charged precursor ions (Zhurov et al. 2013). Experiences from different research groups report that useful spectra for IgG glycopeptides are obtained for precursors below *m/z* 1400 (Alley et al. 2009) or even lower for precursors below 850 Da (Hinneburg et al. 2016). Since the majority of IgG glycopeptide ion signals are in the range around *m/z* 850 or higher (doubly and triply charged), complementing CID with ETD is limited due to the lack of multiply charged precursor ions.

3.4.3.2 Fragmentation of Released Glycans

In tandem MS experiments, glycan modifications reveal additional structural features that would otherwise go unnoticed. Reducing end labeling introduces a chemical tag by which we can easily differentiate between GlcNAc originating from the branches and the GlcNAc from the chitobiose core. This is very informative when assigning fucosylation as core versus antennary. Permethylation of hydroxyl and *N*-acetyl groups improves the ionization efficiency of glycans and enables a more straightforward determination of branching as well as the position of the glycosidic linkage. Major fragments observed in tandem MS experiments of permethylated glycans are ions generated by cleavages of glycosidic bonds at GlcNAc and sialic acid residues. Nevertheless, a significant number of fragments arising from cross-ring fragmentation can also be detected, which allows for a more detailed assignation of linkages between constituent monosaccharides (Morelle et al. 2004).

3.4.4 Ionization Polarity of MS Analysis

Glycans can be analyzed in positive (positively charged ions are measured) or negative ionization mode (negatively charged ions are measured). So far, positive ion mode is used more extensively for glycan analysis as it tends to give better signal intensities, and it is more compatible with the buffered mobile phases and fluorescent dyes (2-AB, ProA) of the widespread HILIC–UPLC–FLR method (Keser et al. 2018; Pabst et al. 2009). Negative ion mode, still not dominantly used for glycan analysis, has a major advantage in the structural analysis of glycans. Fragmentation spectra of negatively charged ions provide far more informative patterns and diagnostic ions. There is no need for any derivatization to gain additional data about glycan structure as cross-ring fragmentations dominate in the negative mode tandem MS experiments of unmodified glycans (Harvey 2020). Cross-ring fragmentations, in contrast to cleavage of glycosidic bonds typical for positive ion mode, produce a number of diagnostic ions highly specific for the branch occupancy (e.g., galactose positioned on 6-arm versus 3-arm) and for the type of linkages between constituent monosaccharides [α(2,6) versus α(2,3) sialic acid linkage] (Harvey 2020).

Nevertheless, negative ion mode is also feasible, if needed, for derivatized glycans where some dyes even increase the ionization efficacy (Pabst et al. 2009). However, some fluorescent tags might change the fragmentation patterns and reduce the level of information in the corresponding fragmentation spectra (Harvey 2020). Negative ion spectra of glycans are more complex and require substantial experience for proper interpretation, making this MS approach somewhat disadvantageous for routine IgG glycosylation analyses.

3.4.4.1 Example of Positive Ion Mode Fragmentation

The main event of positively charged ion fragmentation is the dissociation of glycosidic bond (Conboy and Henion 1992; Huddleston et al. 1993). Figure 3.5 shows a typical tandem MS spectrum of the 2-AB-labeled FA2BG1 structure analyzed by HILIC-UPLC-FLR-MS. Several structural features were successfully deciphered from the combined separation- and MS-based approach. The most abundant ion in Fig. 3.5 at *m/z* 366.13, $[M + H]^+$, is a typical diagnostic ion for galactosylation of the antennae. Additionally, HILIC can successfully separate monogalactosylated isomers with galactose linked to 3-arm versus 6-arm. With these two sets of information, it is possible to assign a galactose linked to 6-arm of the glycan. The ion with the *m/z* value of 488.21, $[M + H]^+$, locates the fucose to the chitobiose core. Reducing end derivatization of the glycan structure with the fluorescent dye marks the innermost GlcNAc and produces a diagnostic ion that annotates the structure as core fucosylated. During the CID of antennary fucosylated structures, it was observed that in certain conditions, an intramolecular fucose transfer between antennae could occur. It is an event characterized by the migration of fucose from its original position during fragmentation, whereby fucose creates linkages with adjacent monosaccharides, producing artifacts rather than diagnostic ions (Hsu and Turk 2004; Kováčik et al. 1995; Wuhrer et al. 2006; Harvey et al. 2002). It is, therefore, important to be cautious when assigning a structure to glycans carrying fucose and to back up the analysis with the appropriate standards. An additional informative fragment is detected at the *m/z* 1056.451, $[M + H]^+$, pointing to the presence of a bisected GlcNAc rather than a third antenna. The rest of the fragments supply information about the sequence and, thereby, about the composition of the analyzed glycan. With all the information from the retention time of the glycan, its fragmentation spectra, and biological origin (human IgG glycan), it is possible to assign the structure as the abovementioned FA2BG1.

3.4.4.2 Example of Negative Ion Mode Fragmentation

As mentioned above, the main advantage of negative ion mode fragmentation is the generation of cross-ring cleavages. It is a type of reaction where two bonds across a single monosaccharide unit are cleaved, yielding A- and X-ions (Fig. 3.4). Figure 3.6 shows the CID MS spectrum of a 2-AB labeled FA2B glycan recorded in negative

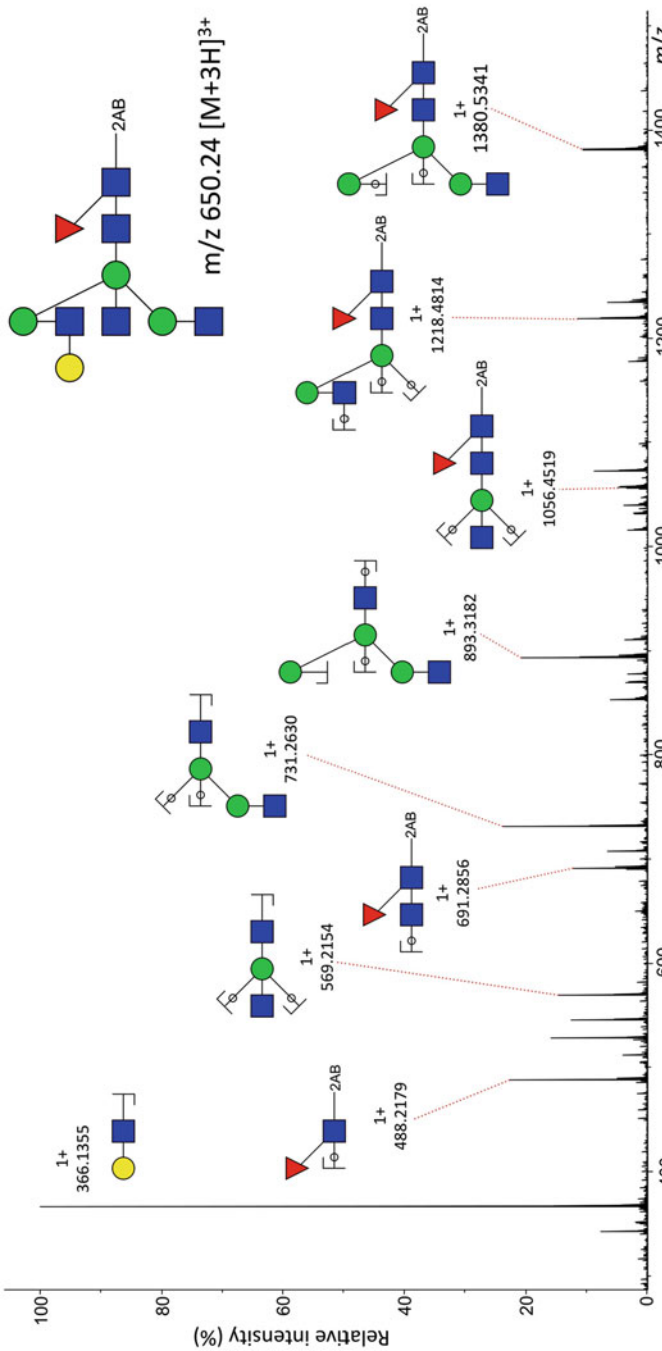


Fig. 3.5 Collision-induced fragmentation (CID) of the triply charged 2-AB labeled core fucosylated, monogalactosylated, biantennary glycan with bisecting GlcNAc recorded in positive mode. Diagnostic $[M + H]^+$ ions: *m/z* 366.13 galactosylation; 488.21 core fucosylation; 1056.45 bisecting *N*-acetylglucosamine (GlcNAc). The rest of the fragments represent proposed fragmentation events. Symbols for monosaccharide units of fragment ion compositions are represented as defined by CFG: blue square (*N*-acetylglucosamine), green circle (mannose), yellow circle (galactose), and red triangle (fucose)

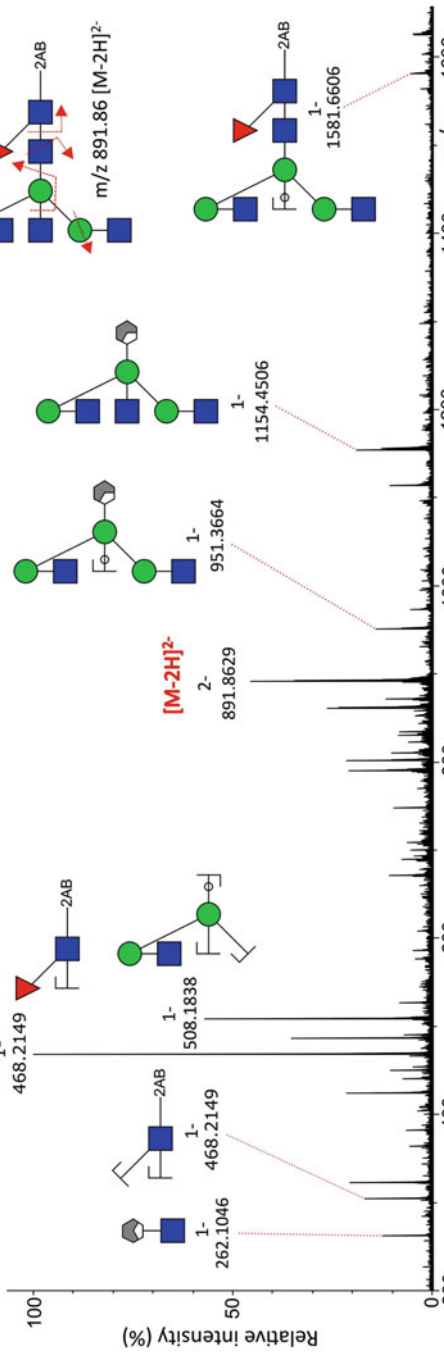


Fig. 3.6 Collision-induced fragmentation (CID) of the doubly charged 2-AB labeled core fucosylated, biantennary glycan with bisecting GlcNAc (FA2B) recorded in negative mode. Diagnostic $[M\text{-}H]^-$ ions: *m/z* 468.21 core fucosylation; *m/z* 508.18 bisected *N*-acetylglucosamine (GlcNAc)—$[D\text{-}221]^-$. The rest of the fragments represent proposed fragmentation events. Symbols for monosaccharide units of fragment ion compositions are represented as defined by CFG: blue square (*N*-acetylglucosamine), green circle (mannose), and red triangle (fucose)

ion mode. The most abundant ion in the spectrum is the $[M-H]^-$ Z-ion with the *m/z* 468.221. It originates from the cleavage of the glycosidic bond between chitobiose core GlcNAcs. Since the reducing end is derivatized with the 2-AB fluorescent dye, we can assign the fucose to the core of the structure. In the fragment spectrum of glycans carrying bisecting GlcNAc, the otherwise present so-called D-ion is missing a result of cleavages between Manβ1-4GlcNAc and Manα1-3Man. The resulting loss of chitobiose core and the 3-arm yields the D-ion neighboured by $[D-18]^-$ formed by neutral loss of water (Harvey et al. 2008; Everest-Dass et al. 2013). Instead, one of the most prominent signals arises from the $[D-221]^-$ ion. It represents the 6-arm occupancy, with the loss of bisecting GlcNAc. In the case of the structure in Fig. 3.5, it is the $[M-H]^-$ fragment with the *m/z* value of 508.18. The main ambiguity in the analyzed FA2B structure arises from its differentiation from the potential isomer FA3. It has the same mass and the same composition as FA2B, and the retention times of two isomers in the HILIC–UPLC analysis are too close to resolve the issue. The only difference between the two isomeric structures is the position of one GlcNAc: is it the third antenna or bisecting GlcNAc? This is exactly the question that can be answered unequivocally with the information from fragmentation in negative ion mode, where the detection of abundant $[D-221]^-$ ion clearly resolves the issue (Fig. 3.6).

3.4.5 Exoglycosidase Digestion Monitored by MS

Glycan sequencing by exoglycosidases uses the specificity of a broad range of enzymes for different monosaccharide types, linkage anomericity, linkage position, aglycon component (proteins, lipids, etc.), and monosaccharide stereoisomerism. Enzymes that remove particular monosaccharides from the non-reducing end of a glycan can be added sequentially to the enzymatic reaction, or they can be premixed and added simultaneously (Dwek et al. 1993). The most accurate and simplest approach is to analyze the structure of a single fractionated and purified *N*-glycan. The approach is also used for the analysis of complex mixtures, for example, a complete IgG *N*-glycome library. The method requires a supporting analytical technique that allows recognition of any possible changes introduced in the sequence of analyzed glycan. Techniques most often used in this type of analysis are separation-based ones which detect signals from fluorescent labels (capillary electrophoresis, ultra-high-performance liquid chromatography) and monitor shifts in retention time reflecting potential digestions of glycosidic bonds. Although not applied as often as the previously mentioned combination, MS techniques can also successfully be applied to monitor the enzymatic reactions. MALDI is an interesting option as it allows, with its speed and simplicity of sample preparation and efficient evaluation of the assay (Matsumoto et al. 2000; Kannicht et al. 2019). There is no need for chromatographic separation, and the combination of the information about applied exoglycosidase with the accompanied monosaccharide mass loss can provide valuable information about sequence and the type of glycosidic linkage.

3.5 Selected Approaches for IgG Glycosylation Analysis

3.5.1 MALDI–MS

Matrix-assisted laser desorption/ionization is a type of laser desorption ionization and one of the most widely used soft ionization techniques. Here, little energy is passed over to the molecule preventing uncontrolled fragmentation (Gross 2004). Compared to the second most widely used soft ionization technique—electrospray ionization (ESI), MALDI mass spectra are less complex to analyze since multiple charges are not common for MALDI ionization (Saba et al. 2002). Studies have demonstrated MALDI application in intact proteins, subclass site-specific, and IgG released *N*-glycans analysis (Huhn et al. 2009). The MS instruments with MALDI source are ideally combined with TOF and FT-ICR mass analyzers, although the latter is better suited for laser desorption ionization (LDI) MS (Gross 2004).

3.5.1.1 Intact Proteins and Glycopeptides

In the top-down and the middle-down approaches, the MALDI in-source decay (ISD) setup was reported as beneficial in the sequence analysis of undigested proteins. The ISD occurs upon transfer of hydrogen radical from the matrix to the radical-sites of the neutral analytes (Takayama 2016). This is achieved by fragmentation of the entire polypeptide chain as a result of hydrogen radical transfer from the matrix (Ayoub et al. 2013). Using MALDI–ISD, IgG can be analyzed in its denatured and reduced form after detergent, DTT, and IAA treatment. Following detergent removal, samples can be spotted on a MALDI target plate and analyzed with an FT-ICR mass spectrometer. Another approach combined IdeS digestion and separate glycosylation analysis of Fab and Fc portion (Tran et al. 2016). In this case, MALDI–ISD also enables the analysis of the N- and C-terminus protein sequence of the heavy chain as an advantage to the classical method, the Edman sequencing, which provides information only about the N-terminus. Additionally, Edman sequencing is limited to up to 50 amino acid residues, but with this method, coverage can be greater than 50 even without protein hydrolysis (Ayoub et al. 2013; Tran et al. 2016).

In the bottom-up approach, prior to MALDI-TOF IgG subclass-specific glycosylation analysis, IgG is usually digested with trypsin. Samples are then desalted, followed by HILIC–SPE glycopeptide enrichment (Selman et al. 2012b). One of the challenges generally arising in the MS analysis, including the MALDI approach, is the difference in the ionization efficiency of peptides and glycopeptides, so relative abundances of IgG subclasses do not always reflect their real abundances in biological samples (Selman et al. 2012b).

3.5.1.2 Released *N*-Glycans

Among other abovementioned approaches aimed at the analysis of released IgG *N*-glycans, MALDI–MS is widely used for this purpose due to its speed, simple sample preparation, and absence of sample separation. This ionization technique offers a possibility to analyze unmodified glycan moieties as well as glycans modified and labeled at the reducing end. Unmodified structures are usually observed in spectra as sodium adducts (Harvey 2001). The reducing end of glycans can be fluorescently labeled to avoid the appearance of intense metal adducts signals in spectra, which can usually be observed after chemical or enzymatic release (Gil et al. 2010). Since the detailed structure and linkage information about glycan species are available only by analysis of native or labeled glycans by MALDI–TOF–MS, separation by normal-phase high-performance liquid chromatography (NP–HPLC) can be introduced before the MALDI–MS analysis. Collected peaks can be treated with high purity exoglycosidases and subsequently analyzed by MS to elucidate the sequence and linkage of glycan moieties (Qian et al. 2007).

Acidic glycans such as sialylated ones are more prone to the production of sodium and potassium adducts, so it is crucial to remove additional salts from the sample before MS analysis (Harvey 2001). One option is resin beads that can be mixed with the matrix (Harvey 2001). Alternatively, cation-exchange resins can also be used for purification before sample application onto the MALDI target (Saba et al. 2002). Dialysis on microfiltration membrane and drop-dialysis are also methods of choice for desalting the samples before applying them onto the target plate and MALDI-Q-TOF analysis (Qian et al. 2007). Besides salts, excess derivatization reagents can be removed in a post-reaction work-up using C_{18} or HILIC cartridges (Gil et al. 2010).

3.5.1.3 Sialic Acid Stability

The sialic acids glycosidic bonds are more prone to hydrolysis than those of other monosaccharides, and chemical derivatization is readily used for stabilization. There are methods reported for both the derivatization of sialic acids for released *N*-glycans and glycopeptides. The different ionization efficiencies of neutral and acidic glycans are the major problem in the analysis of complex glycan mixtures. Various methods can be employed to neutralize charged glycans, such as amidation in mildly acidic conditions or permethylation (Krenkova et al. 2013). With permethylation, sensitivity can also be improved (Harvey 1996). Methyl esterification and amidation have the disadvantage of incomplete modification of α(2,3)-linked sialic acid. As an alternative, acetohydrazide and 1-ethyl-3(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) can be used to treat glycoproteins since aldehydes of the reducing ends of glycans are prone to react with hydrazide. Another problem could be the precipitation and aggregation due to low protein isoelectric point, but so far, that wasn't reported with recombinant mAbs since they have a basic pI (Gil

et al. 2010). A carboxylic activator EDC and 1-hydroxybenzotriazole (HOBt) in methanol or ethanol as a catalyst results in ethyl-esterification and lactonization of sialic acids. This derivatization method was applied on released *N*-glycans resulting in better sialic acid stability and additional information about their linkage since ethyl ester was formed with structures carrying α(2,6)-linked sialic acid (+14.016 Da) and lactone was the result of the reaction of α(2,3)-linked sialic acid with the neighboring galactose (−18.011 Da). For glycopeptides, the situation is different. Dimethylamine was introduced to additionally stabilize sialylated species and also to produce a larger mass difference. The reaction products are dimethylamide with α(2,6)-linked sialic acids (+27.047 Da) and a lactone with α(2,3)-linked sialic acids (−18.011 Da) (De Haan et al. 2015). Besides sialic acid stabilization, these derivatization methods enable a distinction between α(2,3) and α(2,6) linkages, which adds value to the characterization of IgG sialoglycans.

3.5.1.4 Matrix Substances for MALDI–MS

A proper matrix choice plays an essential role in ISD prevention and sialic acid loss (Selman et al. 2012b). Sample and the UV-absorbing matrix are both applied to the target and allowed to co-crystallize. In order to reduce crystal size and improve glycan solubility, recrystallization from ethanol is often applied (Harvey 2001). Among a wide variety of matrices, some of them are commonly used in IgG *N*-glycans analysis, such as 2,5-dihidroxybenzoic acid (DHB) or super-DHB, which contains 10% 2-hydroxy-5-methoxybenzoic acid (Harvey 2001). For glycopeptide spotting, a good performance regarding signal intensity is obtained with α-cyano-4-hydroxycinnamic acid (4-HCCA), either alone or mixed with nitrocellulose. For large proteins and glycoproteins, sinapinic acid is a matrix of choice (Harvey 2001). 4-HCCA has characteristics of a "hot" matrix, such as high proton affinity and extensive analyte fragmentation, and can cause a complete degradation of sialylated glycopeptides (Zauner et al. 2013). Therefore, two "cold" matrices—DHB and 4-chloro-α-cyanocinnamic acid (Cl-CCA)—were tested and enabled detection of intact sialylated glycopeptides, especially in a negative mode. In addition, Cl-CCA showed a better performance in the detection of highly sialylated species compared to DHB. These findings revealed significant benefits of Cl-CCA use in glycopeptides analysis (Selman et al. 2012b).

3.5.1.5 MALDI–MS for High-Throughput and Quantitative Analysis

Protocols for IgG glycosylation analysis by MALDI–MS were optimized for high-throughput applications, and some were also automated (Shubhakar et al. 2016; Bailey et al. 2005). In 2005, a high-throughput platform for MALDI glycopeptide analysis of recombinant mAbs was developed on microplates (Bailey et al. 2005). Hence, purification with C_{18} cartridges can be easily translated to a high-throughput mode, and the MALDI-TOF instrument can be coupled with an MS^n instrument in

order to perform fragmentation on multiple levels and obtain as many details about the analyzed structure as possible (Huang et al. 2017). Furthermore, the high-throughput protocol for *N*-glycan analysis can be automated using HILIC–SPE enrichment, permethylation, and liquid–liquid extraction, followed by MALDI-TOF–MS (Shubhakar et al. 2016).

Processing a large amount of data is always a challenge, especially in high-throughput settings. Data analysis, extraction, and quantification can be done using a number of commercially available software packages or with specifically developed tools distributed under free software licenses, like MALDIquant, Mass-Up, or MassyTools (Jansen et al. 2015; Gibb and Strimmer 2012; López-Fernández et al. 2015).

MALDI–MS techniques are considered semi-quantitative since glycan species ionization is structure-dependent (Saba et al. 2002).

3.5.2 LC–MS for IgG Glycosylation Analysis

3.5.2.1 Coupling LC to MS for Enhanced Separation and Structural Characterization

Glycans show a high degree of structural heterogeneity with various linkage arrangements and monosaccharide branching within the molecule. Such complexity causes difficulties in isomer separation and makes structural analysis challenging. Often a single analytical technique cannot untangle complex mixtures of glycans and define their structural properties (Wuhrer et al. 2009). In order to unravel the complete glycan structure, multiple approaches should be combined to obtain complete information on linkage specificity, composition, isobaric, and isomeric structures (Mendez-Huergo et al. 2014; Miura and Endo 2016; Abrahams et al. 2018).

Although mass spectrometry proved to be a powerful tool for protein glycosylation analyses due to its high sensitivity, speed, and robustness, methods that rely only on accurate mass are unable to distinguish structural isomers (Zaia 2010). Besides structural isomers of released glycans, protease digested glycopeptides can also form various isomeric peptide-glycan combinations. Since antibodies can have one (IgG) to six (IgE) Fc glycosylation sites, there is a large number of possible peptide-glycan combinations that produce the same mass (Arnold et al. 2007).

Mass spectrometers equipped with MALDI sources often require the derivatization of sialic acids to avoid their in-source decay (Luo et al. 2009). On the contrary, in-source fragmentation of glycans can be easily controlled on ESI instruments (Wuhrer et al. 2009). Possible loss of sialic acids or a complete arm from the glycan core can be efficiently diagnosed and prevented by adjusting ESI source voltages (Huhn et al. 2009; Stadlmann et al. 2008). Another way to decrease in-source fragmentation is the use of buffered solutions. However, this also decreases signal intensities when compared to acidic aqueous solvents (Huhn et al. 2009). Negatively charged glycans containing sialic acids have a low ionization efficiency and are

underrepresented in positive mode MALDI analysis. Similar holds true for native or glycans tagged with 2-AB in a negative ion MALDI mode. In this case, again, neutral and charged glycans in both ESI–MS polarities show a higher ionization efficiency and give more representative MS spectra when compared to MALDI–MS (Wuhrer et al. 2005). Ionization processes within ESI sources used for LC–MS predominantly form multiply protonated species, which, in positive mode collision-induced dissociation analysis, do not form cross-ring fragments required for detailed structure determination. Higher-order tandem MS methods should be used to untangle the structural features of glycans or glycopeptides.

Released glycans have 1–2 orders of magnitude lower ionization efficiency when compared to peptides (Karas et al. 2000). For this reason, glycans are usually derivatized to increase ionization efficiency. Furthermore, in complex mixtures, ionization of glycopeptides or glycans tends to be suppressed by peptides through competitive ionization and detector saturation (Luo et al. 2009; Hu and Mechref 2012). Therefore, using LC coupled to MS is beneficial for separating samples with potentially limited availability (Luo et al. 2009).

An efficient way to distinguish isomeric structures is by coupling various liquid chromatography methods with mass spectrometry (LC–MS). LC–MS plays an important role in the separation of isomeric structures indistinguishable for MS. Accordingly, a common liquid chromatography method for separating enzymatically released glycan mixtures includes HILIC and PGC (Mauko et al. 2012; Michael and Rizzi 2015; Ruhaak et al. 2009; Saldova et al. 2014). Reversed-phase chromatography is routinely used for the separation of IgG glycopeptides (Selman et al. 2012a), antibody fragments produced by complete or incomplete proteolysis with various proteases, heavy chains obtained by reduction of antibody, and complete intact antibodies (Zhang et al. 2014). Derivatized glycans released from protein can also be analyzed using RP–LC–MS (Abrahams et al. 2018; Sinha et al. 2008). Various mass analyzers such as time-of-flight detectors, triple quadrupoles, ion traps, Orbitraps, and IT-FCRs can be coupled with LC.

3.5.2.2 HILIC–UHPLC–MS

By this approach, a mixture of charged, highly hydrophilic, and uncharged glycans can be easily retained and separated on the column. Glycans can be analyzed either underivatized or derivatized with fluorescent dyes or other reagents. This technique was introduced by Alpert in 1990 and is widely used in various glycomic studies (Reusch et al. 2015a, b; Alpert 1990). HILIC is considered a variant of normal-phase liquid chromatography (NP-LC) because it contains a stationary phase that is more polar than the mobile phase (Buszewski and Noga 2012). Nevertheless, mobile phases used for HILIC differ from solvents used in NP-LC (Hemström and Irgum 2006). Glycans are separated in a gradient changing from a high percentage organic solvent used for binding glycans to the column to a high percentage of aqueous phase (buffer) to separate and elute glycans (Buszewski and Noga 2012). Retention times of analytes on HILIC stationary phases are affected by various interactions,

which include hydrogen bonding, ionic interactions, and dipole-dipole interactions (Wuhrer et al. 2009). Separation is achieved through the interactions between the thin layer of water coating the polar adsorbent and the mobile phase that contains an organic solvent. The salt concentration and pH are important factors governing charged stationary phase surface properties (Veillon et al. 2017).

There are various stationary phases for HILIC columns, of which non-ionic amide-modified columns are mostly used for glycan separation (Wuhrer et al. 2009). Since there is no mass difference between structural isomers, coupling HILIC to MS can further resolve a number of isobaric structures (Butler et al. 2003). Mobile phases for HILIC separations are compatible with mass spectrometers because elution of glycans from the column occurs at a low percentage of buffer containing volatile salts and a high ratio of organic solvent, which enables its relatively easy evaporation and ionization (Wuhrer et al. 2009).

HILIC–ESI–LC–MS is suitable for the analysis of native glycans as well as glycans reduced into alditols or labeled with fluorescent or UV-absorbing dye (Wuhrer et al. 2005). Besides the benefits of high sensitivity for fluorescence detection, fluorophores (see Table 3.3) also increase ionization efficiency and thus the sensitivity of MS detection. Detailed HILIC-MS analysis of commercially available mAb (IgG4) and IgG from human serum was described by Zhao et al. (2016). Fab and Fc fragments were enzymatically separated using specific proteases such as IdeS and further individually analyzed to obtain information on Fab and Fc-specific glycan profiles (Janin-Bussat et al. 2013).

Besides amide/amine HILIC columns, zwitterionic HILIC (ZIC–HILIC) columns can also be used for the separation of released glycans (Mauko et al. 2012). ZIC–HILIC columns showed capability for separation of 2-AP derivatized glycans from human serum IgG, while ZIC–HILIC coupled with ESI–MS can be used for separation and structural elucidation of reduced glycans from mAbs (Takegawa et al. 2006; Mauko et al. 2011). Mauko et al. showed that the ZIC–HILIC column displayed similar results when compared to amide HILIC separation but with a shorter separation time. Also, the use of acetic acid instead of ammonium formate buffer enabled easier coupling to MS (Mauko et al. 2012).

Besides released antibody glycans analysis, HILIC columns can be used for the separation of glycopeptide digests. Although tandem MS analysis of released glycans can give complete information on glycan structure, it does not give any information about protein and glycosylation site. HILIC-MS of glycopeptides provides information on peptide backbone carrying glycan and site-specificity of glycosylation (Wuhrer et al. 2009). Historically, HILIC separation has been traditionally part of 2D chromatography, where the first dimension was RP separation of peptides followed by HILIC separation, which enabled enrichment and separation of glycopeptides (Wuhrer et al. 2009; Zhang and Wang DI c. 1998). HILIC-MS can be performed at the nanoscale and capillary scale, providing femtomole range sensitivity (Wuhrer et al. 2005). IgG tryptic digests separated on ZIC–HILIC column results in the separation of glycopeptides based both on peptide backbone and charge of attached glycans (Takegawa et al. 2006).

3.5.2.3 RP–LC–MS

The most common RP–LC–MS setup includes an RP pre-column (trapping column) that enables online desalting and sample preconcentration, an analytical column, and a Q-TOF instrument equipped with an ESI ion source (Stadlmann et al. 2008). Formic acid is a typical additive in aqueous solvents in gradient separations with increasing organic solvent content (acetonitrile, ACN) (Wuhrer et al. 2007b; Hirayama et al. 1998; Takakura et al. 2014). Trifluoroacetic acid (TFA) can be used instead of formic acid to prevent separation of the same peptides carrying charged and neutral glycans; however, it has a strong suppressive effect on ionization (Shou and Naidong 2005). In order to overcome this problem, propionic acid can be introduced after the analytical column, either using a tee connector or sheath flow ESI sprayer (Selman et al. 2012a).

Endopeptidases, such as pronase, Lys-C, Glu-C, and the most widely used trypsin, are applied for the digestion of immunoglobulins (Huhn et al. 2009). The resulting glycopeptides are combinations of various peptide sequences and attached glycan structures yielding sometimes indistinguishable mass isomers. In the case of trypsin-digested human IgG, three Fc glycopeptides can be separated using RP-LC based on differences in their peptide sequences: IgG1 (EEQY*N*STYR), IgG2 (EEQF*N*STFR), and IgG4 (EEQF*N*STYR). Tryptic peptides from IgG3 allotypes present in Caucasian populations (EEQF*N*STFR) have amino acid composition and sequence that are identical to the IgG2 peptide. On the other hand, tryptic peptides from IgG3 allotypes predominant in Asian and African populations (EEQY*N*STFR) have a different sequence but the same amino acid composition as the IgG4 peptide. In both cases, RP–LC–MS is unable to separate peptides with the same amino acid content, which is the major drawback of this approach (Dard et al. 2001). The subclasses IgG2 and IgG4 or IgG3 and IgG4 thus have to be quantified together.

Source settings considerably affect the analyte charge state. Tryptic IgG glycopeptides typically ionize as doubly, $[M + 2H]^{2+}$, and triply, $[M + 3H]^{3+}$, charged protonated species (Stadlmann et al. 2008; Wuhrer et al. 2007b; Olivova et al. 2008). High concentrations of coeluting peptides can suppress the ionization of targeted glycopeptides, while the measurement of glycopeptides in complex samples is complicated by different ionization efficiencies. Also, protein digests can include peptides or incompletely digested (glyco)peptides with masses overlapping with targeted glycopeptides which additionally complicates the analysis. For these reasons, it is common to use purified antibodies, which are enriched and desalted before digestion (Huhn et al. 2009).

Glycopeptide analysis of other antibody classes is more challenging than that of IgG. The presence of multiple glycosylation sites often requires the use of more than one protease to obtain glycopeptides of optimal length. An LC–ESI–MS/MS method for the analysis of recombinant secretory IgA complexes using digestion with trypsin followed by Glu-C has been described by Paul et al. (Paul et al. 2014; Huang et al. 2015). Associations of effector function IgA subclasses with their glycosylation profiles were recently demonstrated by Steffen et al. (2020). Similar glycosylation

analysis of IgM showed the presence of high-mannose and complex glycans on the heavy chain of the molecule (Loos et al. 2014). Immunoglobulin E, an antibody with six glycosylation sites on each heavy chain mainly occupied by high-mannose and complex-type glycans, can be treated with trypsin, proteinase K, or chymotrypsin to obtain optimal glycopeptides from all glycosylation sites (Plomp et al. 2014).

Reversed-phase liquid chromatography coupled to mass spectrometry is an alternative method for analysis of released glycans, in which separation is achieved through non-covalent interactions with non-polar stationary phases such as C_8 and C_{18} (Vreeker and Wuhrer 2017). There are numerous C_{18} stationary phases with various influences on retention times. One of the advantages of RP-LC is the high compatibility of solvents with MS because they include water, acid (typically 0.1% formic acid), and organic solvent (ACN). Glycans are bound to the stationary phase in the high aqueous phase, with separation occurring at a slightly increased percentage of organic solvent. Native glycans, due to their hydrophilic nature, are not retained on RP columns even under high-salt and low-pH conditions (Fan et al. 1994). Therefore, binding glycans to alkyl chains of the C_{18} column requires derivatization to increase their hydrophobicity. Retention times and separation are highly dependent on the stationary phase, mobile phase, and hydrophobicity of derivatives used. Permethylation is a commonly used derivatization technique preceding glycan analysis on RP–LC due to a significant increase in hydrophobicity. Also, permethylated glycans tend to have higher ionization efficiencies in ESI sources and increased stability of attached sialic acids (Ruhaak et al. 2010a; Vreeker and Wuhrer 2017). Various studies demonstrated the use of RP–LC separation of permethylated IgG glycans (Zhou et al. 2016; Ritamo et al. 2013). However, the presence of side reactions, which are especially favored with low sample amounts, causes problems with the reproducibility of this method (de Haan et al. 2020; Kang et al. 2005). Various labels can be used in RP–MS analysis of IgG *N*-glycans. The most commonly used label for glycans, 2-AB, slightly increases hydrophobicity and requires long runs to achieve separation (Chen and Flynn 2007; Adamczyk et al. 2014). This means that charged glycans labeled with 2-AB are poorly separated on RP-LC columns, which can be fixed by the addition of ion-pairing agents (Higel et al. 2013; Melmer et al. 2011). On the other hand, 2-AA provides a good ionization efficiency, especially for negative mode MS, but also enables separation of glycans based on their type, presence of core fucose, and sialic acids (Higel et al. 2013, 2014). Besides 2-AA, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) is another negatively charged dye convenient for a negative mode MS analysis (Higel et al. 2013). Finally, 2-aminopyridine (2-AP) has the lowest hydrophobicity resulting in rapid eluting glycan peaks. However, it is less compatible with MS since higher amounts of hydrophilic solvent are required (Pabst et al. 2009).

The separation of structural isomers using RP represents a challenging task. Isomeric complex glycan structures generate similar interactions with the stationary phase. The differences between such isomers are not large enough to cause shifts in hydrophobicity which could significantly impact retention properties and achieve baseline separation. Therefore, the most common way to separate glycans on RP is by using shallow linear gradients or even isocratic runs, which take a significant

amount of time and ultimately provide lower peak capacities, i.e., the largest number of equally resolved peaks that can be fit into the separation interval (Melmer et al. 2011). Because of the abovementioned issues, the separation of IgG *N*-glycan isomers is mainly done using HILIC and PGC stationary phases (Veillon et al. 2017).

3.5.2.4 PGC for Enhanced Isomeric Glycan Separation

Porous graphitized carbon liquid chromatography (PGC–LC) is a high-resolution method for the separation of complex glycan mixtures because of its ability to efficiently separate isomeric glycan structures (Abrahams et al. 2018). Glycans bind to PGC through various hydrophobic, polar, and ionic interactions (Fan et al. 1994; Pabst and Altmann 2008a). Additionally, the planarity of graphite type carbon used for the stationary phase in PGC columns enables the separation based on the three-dimensional structure of the molecule (West et al. 2010). On the contrary to RP–LC, polar analytes are strongly retained on the column, which enables the analysis of native, reduced, labeled, or permethylated glycans. Mobile phases used for PGC include organic solvents and aqueous solutions containing acids, bases, or volatile buffers, which makes this separation method compatible with MS (Ruhaak et al. 2009).

PGC–LC–ESI–MS is commonly used for detailed structural analysis of underivatized glycans due to its ability to separate anomers (Abrahams et al. 2018). Although glycan permethylation can be used to increase ionization of sialylated glycans, this derivatization results in inadequate separation, which can be solved by increasing column temperature (Costello et al. 2007; Zhou et al. 2017). The mobile phase choice is crucial for proper separation since its pH and buffer capacity affect the retentivity on the column by determining the ionization state of both the stationary phase and the analyte in solution (Bapiro et al. 2016). During the PGC–LC run, larger high-mannose glycans elute first, while multi-sialylated glycans are strongly retained. Better retention of sialylated glycans can be achieved by using ion-pairing agents such as TFA (Melmer et al. 2011). Native and derivatized glycans will dominantly produce proton adducts, followed by less abundant sodium, potassium, and ammonium adducts (Pabst and Altmann 2008a; Zhou et al. 2017). However, PGC columns are susceptible to contamination which causes column deterioration and affects the retention of analytes (Bapiro et al. 2016). There are also reported interferences between the electric field of the ESI source with the separation on PGC (Carrasco et al. 2001). The solution for this problem was proposed by Pabst et al., who reduced it by the electrical grounding of the column. This research group also presented the importance of ionic strength on the peak shape of charged glycans and studied the effects of solvent, temperature, and ion polarity on the detection and elution of charged glycans. Changes in pH did not affect ionization efficiency in both polarities, although increased retention time of charged glycans was observed. Finally, a high concentration of organic solvent was found to be required to obtain better intensities for charged glycans in negative mode (Pabst and Altmann 2008b). Use of PGC–LC–MS for glycosylation analysis of

polyclonal human IgG and commercial mAb was described by Stadlmann et al., demonstrating separation of neutral and sialylated glycans released from antibodies within a single run (Stadlmann et al. 2008). Abrahams et al. obtained glycosylation profiles of various glycoproteins, including IgG and IgA, and demonstrated the need for a PGC retention time library (Abrahams et al. 2018; Stadlmann et al. 2008). Glycopeptides can also be separated on PGC. Nevertheless, there are reports of difficulties eluting sialylated glycopeptides from the column (de Haan et al. 2020). Antibody glycopeptide analysis using PGC published by Huang et al. included comprehensive site-specific glycosylation analysis of Pronase E-derived glycopeptides originating from human colostrum IgA complexes (Huang et al. 2015).

3.5.2.5 Anion Exchange LC–MS

Anion exchange separation techniques can be utilized for the separation of glycans since they are weak acids (Cataldi et al. 2000). High-pH anion exchange chromatography (HPAEC) coupled with MS can be used to obtain glycan structural information. Stationary phases used in HPAEC columns are polymer-based and are stable in a wide pH range. Advantages of using this method were presented by Maier et al., who described structural assignment and quantification of IgG glycans using HPAEC coupled to MS (Maier et al. 2016). Usually, glycans are separated under alkaline conditions, and there is no need for derivatization. Retention on the column is primarily affected by analyte formal charge, size, linkages, and composition (Veillon et al. 2017). The coupling of HPAEC with MS can be problematic due to the high-salt concentrations, which significantly suppress the ionization of glycans. A desalter such as a microfluidic membrane suppressor can be coupled to an alkaline mobile phase to enable sodium-free ionization conditions. Although it is not as widely used as the other methods, its separation mechanism and selectivity make it an interesting alternative to PGC-, RP-, and HILIC-based chromatography (Maier et al. 2016).

3.5.2.6 Challenges of Miniaturization

Separation, throughput, and sensitivity can be drastically altered by changing column variables such as diameter, length, and particle size of stationary phase (Vreeker and Wuhrer 2017). C_{18} nano-LC–MS is a commonly used method for peptide separation. Even in the case of glycan analysis, this method gives higher sensitivity, separation efficiency, and resolution when compared to conventional LC-columns-based methodology (Veillon et al. 2017). Compared to traditional LC–MS, analysis of 2-AB labeled glycans analyzed on nano-LC–MS systems were reported to have ten times higher sensitivity. When compared to MALDI–TOF–MS of NP-LC fractionated and collected peaks, the increase in sensitivity is 100-fold. Nevertheless, nano-LC systems display higher instability of retention times between injections. In the case of fluorescently labeled glycans, this can be easily corrected using a dextran

ladder retention time recalibration (Wuhrer et al. 2005; Royle et al. 2002; Rudd et al. 2001). In addition, nano-LC chromatography runs are significantly longer and, due to smaller inner diameters of capillaries and columns fluidic parts, are more susceptible to clogging. Moreover, tight fluidic connections must be considered because even the smallest dead volume can severely impact retention times and peak shape. A limited amount of sample can be injected, but this can be solved by using trapping columns on which higher flow rates and, therefore, larger sample volumes can be applied (Vreeker and Wuhrer 2017). Due to the aforementioned issues limiting nanoscale chromatography, antibody glycosylation analyses are often performed on micro and capillary-LC scale (Huhn et al. 2009; Stadlmann et al. 2008; Rehder et al. 2006; Huang et al. 2005; Harazono et al. 2008).

3.5.3 Capillary Electrophoresis-Mass Spectrometry

Capillary electrophoresis (CE) as a separation technique for IgG glycosylation analysis can be used independently or coupled to MS detection (Volpi and Maccari 2013). The separation of analytes is based on charge, size, and shape properties (Volpi and Maccari 2013), and the main advantage is the separation of positional isomers (Zaia 2013). Several CE techniques are available: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), isotachophoresis (ITP), micellar electrokinetic capillary chromatography (MECC/MEKC), microemulsion electrokinetic chromatography (MEEKC), nonaqueous capillary electrophoresis (NACE), and capillary electrochromatography (CEC) (Volpi and Maccari 2013). However, the number of CE techniques that can be coupled to MS is somewhat limited since solvents that can be used for efficient CE separation are, in that case, restricted to volatile buffer systems. Therefore, CZE is the technique that is most often a part of CE–MS (Huang et al. 2017). CE–MS coupling can be done in an online or an offline setup. The two most common ionization techniques used are ESI (positive and negative ion mode) preferentially for the online coupling and MALDI for the off-line setup.

The online coupling has been done with different types of interfaces: coaxial sheath flow interface, the liquid junction, or sheathless interface (Zhong et al. 2014; Gennaro and Salas-Solano 2008). Coaxial sheath flow is used at the distal end of the capillary with a flow of a few μL/min which can cause a dilution of analytes migrating from the capillary. To overcome this issue and provide sufficient flow (below 0.5 nL/min) for the MS source at the same time, a liquid junction interface can be used (Zaia 2013). Sheathless interface is used in normal polarity separation, where the use of buffer at high-pH values promotes ionization. On the other hand, the introduction of acidic buffers is beneficial for reverse polarity analysis in combination with the coating of capillary and pressure assistance (Bindila et al. 2005). Bare fused silica capillaries are usually used to reduce the possibility of capillary coating bleeding into the MS source. Interaction between glycopeptides

and the walls of uncoated capillaries can occur, so a washing procedure can be introduced after each CE–MS sample injection (Zaia 2013).

Analyses of both underivatized and derivatized glycans were reported in the literature. Jayo et al. optimized conditions for underivatized glycan analysis in reversed polarity mode and negative mode ESI–MS (Jayo et al. 2014). Besides APTS as the most common label used for derivatization via reductive amination, Fmoc-Cl is also an attractive option for derivatization prior to MS analysis. Excess of labeling reagent is usually removed by solid-phase extraction (SPE) prior to the analysis (Nakano et al. 2009). SPE can also be part of the inline SPE–CE–MS system mainly used for cations analysis. It can also be beneficial in the enrichment and analysis of the anions of strong acids like APTS-labeled glycans. These analytes showed 1000 times higher sensitivity in CE–LIF analysis compared to CE–MS. When applying an in-line SPE-CE–MS system for separation of APTS-labeled glycans in acidic background electrolyte (BGE), 800 times lower concentration of analytes can be used and still provide comparable intensities to standard CE–MS (Jooß et al. 2014). For ESI, derivatized glycans have the advantage of better ionization compared to native neutral oligosaccharides lacking easily ionizable functional groups. Specifically, APTS-labeled glycans can maintain a negative charge at low-pH values (Gennaro et al. 2006). Even though TOF mass analyzers were preferentially used in CE–MS coupling, a method using drift tube ion mobility (DTIM) was developed to analyze derivatized and native *N*-glycans. Accordingly, the CZE–DTIM–MS setup showed excellent separation and reproducibility (Jooß et al. 2019).

For the analysis of intact mAbs, a microfluidic CE–ESI–MS strategy was applied and was successful in the separation of intact mAbs charge variants. The main challenges arising here were to prevent analyte adsorption and to enhance electrophoretic mobility differences. High ionic strength buffers, surfactants, and additives in BGE have that role, and often triethylenetetramine and ε-aminocaproic acid are added for that purpose. However, the problem of their incompatibility with ESI remains. In a microfluidic strategy, uniform and stable aminopropyl silane-polyethylene glycol surface coating was used, which eliminated additives, enabled MS-compatible BGE, and resulted in the highly efficient separation of intact mAbs (Redman et al. 2015). Capillary electrophoresis offers many opportunities for glycan and glycopeptide analysis, even more as new solutions emerge regarding the compatibility and improvement in coupling CE techniques to MS detection.

3.6 Perspectives

An accelerating transition from traditional to precision medicine has transformed how fundamental research translates from lab bench to patient bedside care and the other way round. The increasingly popular comprehensive multi-omics approaches for studying biological fingerprints require the fast development of novel analytical methods followed by big data mining, integration, and network analysis. These

breakthroughs can reveal hidden insights into patterns and relationships entangling all biological organization levels from the molecular and single-cell to the population level on the pathway to individualized therapy.

Mass spectrometry is no exception here, although its implementation into the clinic was slow due to complex instrumentation, lengthy protocols, and challenging interpretation of the results with shadowy meaning to the medical practitioners. Miniaturization, more robust analytical workflows, sample multiplexing, and technical advancements in the field of chromatography made mass spectrometry more tempting for widespread use in protein post-translational modifications analysis, including glycosylation. In this chapter, a concise methodological overview of IgG—a central immune system glycoprotein—*N*-glycosylation analysis was given. In the past decade, extensive large-cohort studies revealed changes in IgG glycosylation to be correlating with numerous pathological states. This promisingly positions immunoglobulin glycans as potential biomarker candidates and paves the way for the advent of new disciplines such as pharmacoglycomics or glycoimmunology, all made possible by the invention of a mass spectrometer.

Compliance with Ethical Standards

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Ethical Approval: This book chapter does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 4
Capillary (Gel) Electrophoresis-Based Methods for Immunoglobulin (G) Glycosylation Analysis

Samanta Cajic, René Hennig, Robert Burock, and Erdmann Rapp

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Abstract The in-depth characterization of protein glycosylation has become indispensable in many research fields and in the biopharmaceutical industry. Especially knowledge about modulations in immunoglobulin G (IgG) *N*-glycosylation and their effect on immunity enabled a better understanding of human diseases and the development of new, more effective drugs for their treatment. This chapter provides a deeper insight into capillary (gel) electrophoresis-based (C(G)E) glycan analysis, addressing its impressive performance and possibilities, its great potential regarding

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S. Cajic
Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

R. Hennig (✉) · E. Rapp
Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

glyXera GmbH, Magdeburg, Germany
e-mail: r.hennig@glyxera.com

R. Burock
glyXera GmbH, Magdeburg, Germany

M. Pezer (ed.), *Antibody Glycosylation*, Experientia Supplementum 112,
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real high-throughput for large cohort studies, as well as its challenges and limitations. We focus on the latest developments with respect to miniaturization and mass spectrometry coupling, as well as data analysis and interpretation. The use of exoglycosidase sequencing in combination with current C(G)E technology is discussed, highlighting possible difficulties and pitfalls. The application section describes the detailed characterization of *N*-glycosylation, utilizing multiplexed CGE with laser-induced fluorescence detection (xCGE-LIF). Besides a comprehensive overview on antibody glycosylation by comparing species-specific IgGs and human immunoglobulins A, D, E, G, and M, the chapter comprises a comparison of therapeutic monoclonal antibodies from different production cell lines, as well as a detailed characterization of Fab and Fc glycosylation. These examples illustrate the full potential of C(G)E, resolving the smallest differences in sugar composition and structure.

Keywords Capillary gel electrophoresis · *N*-glycosylation · Biopharmaceuticals · Immunoglobulin · IgA · IgD · IgE · IgG · IgM · Monoclonal antibody · mAb · APTS · xCGE–LIF · CE–MS · Chip–CE · Exoglycosidase

Abbreviations

APTS	8-aminopyrene-1,3,6-trisulfonic-acid
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CHO	Chinese hamster ovary
CQA	Critical quality attribute
CZE	Capillary zone electrophoresis
DB	Database
EOF	Electroosmotic flow
ESI	Electrospray ionization
Fab	Fragment antigen binding
Fc	Fragment crystallizable
Fuc	Fucose
Gal	Galactose
GlcNAc	*N*-acetylglucosamine
GU	Glucose units
HAGR	Hepatic asialo-glycoprotein receptor
HILIC	Hydrophilic interaction liquid chromatography
HMOS	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
LIF	Laser-induced fluorescence detection
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption ionization with time-of-flight MS

Man	Mannose
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTU	Migration time unit
MTU″	Double aligned migration time units
Neu5Ac	*N*-acetyl-neuraminic acid
Neu5Gc	*N*-glycolyl-neuraminic acid
NMR	Nuclear magnetic resonance
Sia	Sialic acid
SNFG	Symbol nomenclature for glycans
UPLC	Ultra-performance liquid chromatography
xCGE-LIF	Multiplexed capillary gel electrophoresis with laser-induced fluorescence detection

4.1 Historical Background

Electrophoresis was born more than 200 years ago (Ruess 1809), even long before the concept of chromatography was described. However, it took about 150 more years before the use of capillaries was introduced (Hjertén 1967). From the late 1960s, it took an additional decade to establish capillary electrophoresis (*CE*) as it is most widely known today, enabling separations that seemed unattainable at that time (Jorgenson and DeArman Lukacs 1981; Jorgenson and Lukacs 1981a, b). The increased efficiency and the amazing separation capabilities together with short analysis times, induced a growing interest among the scientific community. Since then, CE has constantly been improved and has become an important tool in the analysis of a wide class of compounds, from small ions over amino acids and peptides/proteins to large DNA fragments (Saraswathy and Ramalingam 2011). However, the employment of CE for glycan analysis lagged behind other commonly used analytical techniques. Attempts to use CE for glycan analysis started in the 1990s, but they were rather humble and not broadly embraced—neither by the glycoscientific community nor by industrial laboratories. Today, 40 years since the potential of CE was recognized, the advantages of the method for analysis of glycans are becoming increasingly obvious and appreciated. Ready-to-use CE methods and kits on the market (glyXera 2020; Thermo Fisher Scientific 2018; Ludger 2018; ProZyme 2018; SCIEX 2018; PerkinElmer 2018) enable fast and robust comparison of glycan profiles, which makes the technology more routine in academic research and industry. In the following sections, we will emphasize some of the reasons why CE is gaining popularity and underline why this technology is attractive for the biopharmaceutical industry, especially regarding the analysis of immunoglobulin (*Ig*) glycosylation. Additionally, some current and future challenges, as well as possible future methodological developments, will be addressed.

4.2 Background: Principles of Capillary (Gel) Electrophoresis (C(G)E)

Capillary electrophoresis is the umbrella term for various capillary electrophoretic separation techniques and methods, such as capillary zone electrophoresis (*CZE*) and capillary gel electrophoresis (*CGE*). The differential migration of charged molecular species (ions) in a narrow capillary (25–75μm) containing an electrolyte solution under the influence of high voltage (usually 10–30 kV, generating an electric field) is the basis for separation by CE (Watson 2012). An analyte is attracted to either the anode (positive electrode) or the cathode (negative electrode), and its movement (electrophoretic mobility) in an electric field results from both electrical and frictional force contributions. The movement of a charged analyte through a conductive solution is dependent on the charge of the analyte and the magnitude of the applied electric field. Additionally, a frictional force will impede the movement induced by the electrical field to a certain extent, dependent on the viscosity of the electrolyte solution and the analyte radius/molecular size or shape. Thus, the mobility and, therefore, the separation is based on the size-to-charge ratio of the analytes. Besides electrophoretic velocity, electroosmotic flow (*EOF*) is influencing the movement of the analyte through the capillary. EOF is the bulk liquid motion in a capillary when a high voltage (electric field) is applied. In a capillary composed of fused silica, the surface possesses negative charges (SiO^-) over a wide pH range (pH 4–12). This negatively charged surface attracts positively charged electrolytes from the running buffer. Along an electric field, these cations (enriched at the capillary wall) move towards the cathode. Due to solvation and liquid viscosity, these cations drag the surrounded bulk solvent with them along the capillary axis, creating a net flow toward the cathode, the EOF. Thus, all analytes, irrespective of their charge, are pushed in one direction, enabling simultaneous separation and detection of cationic (EOF + ion migration = fastest), neutral (EOF = middle) and anionic (EOF − ion migration = slowest) analytes (Holland et al. 1997). Although this is rather a rough summary of the separation mechanism, the reader should recognize that the parameters involved in optimizing the technique to produce separation are very complex. However, the effort pays off as very small changes in molecular structure can lead to quite marked differences in migration if variables are carefully balanced. There is a large volume of literature regarding CE separations of glycans exploiting a variety of possible setups, methods, modes, and parameters (e.g., capillary material, dimensions, and surface coating, voltage, current, polarity, temperature, buffer type and concentration, pH, additives/modifiers, injection technique, detection mode, etc.) that is beyond the scope of this review (Campa and Rossi 2008; Ruhaak et al. 2010a; Lu et al. 2018). However, due to a boom of applications in the field of glycan analysis and particular interest in the biopharmaceutical industry, one technique deserves to be further dissected—capillary gel electrophoresis (CGE).

After providing the complete genetic blueprint of human life (Collins et al. 2004), CGE-based Genetic Analyzers (DNA analyzers) found their application in the field of glycan analysis, with a more complex challenge but a new hope of deciphering the

glycome (Schwarzer et al. 2008; Laroy et al. 2006; Callewaert et al. 2001; Ruhaak et al. 2010b). These instruments combine four great advantageous features, which make them very powerful analytical tools in glycoscience. First, in CGE, the abovementioned EOF is typically completely suppressed by employing a neutral capillary (permanently or dynamically coated) filled with a gel mesh polymer. The use of such a gel buffer increases the viscosity of the electrophoresis medium. Consequently, due to the role of viscosity in frictional drag, this increases the time analytes spend in the capillary (decrease of electrophoretic mobility), while decreasing diffusion and thereby improving the separation (Watson 2012; Guttman et al. 1994; Luo et al. 2010). Thanks to this first advantageous feature, glycans are separated based on mass-to-charge ratio and molecular size/shape (hydrodynamic volumes) with high resolution (Guttman et al. 1996a; Guttman and Herrick 1996). Second, the large surface-to-volume ratio of narrow capillaries enables a very good heat transfer. This in turn raises the possibility to apply very high electric fields, which increases the electrophoretic mobility and consequently decreases the analysis time. Third, besides its high separation efficiency in a short time, this method is also attractive due to the possibility to employ laser-induced fluorescence (*LIF*) detection, known for its impressive sensitivity (low attomole range) (Guttman 1996; Hennig et al. 2011a). The fourth advantage is the possibility to employ a multi-capillary format (multiplexing), incorporating up to 96 capillaries in parallel, so that hundreds of samples can be measured by CGE per day (Mittermayr et al. 2013). As a result, modern multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (*xCGE-LIF*) instruments offer an amazing high-throughput, separation performance and sensitivity. Another attractive option provided, e.g., by Applied Biosystems Genetic Analyzers, is a recording of up to six different fluorescent dyes (in six independent detection traces/channels) simultaneously within one run (all excited by only one laser). As described later, this special feature has been exploited for internal alignment of migration times (glycans and internal base pair standard labeled with different dyes), giving long-time stable migration times (Callewaert et al. 2001; Laroy et al. 2006; Schwarzer et al. 2008; Reusch et al. 2014). The basic setup of a Genetic Analyzer ((x)CGE-LIF system) and the principle of glycan separation is depicted in Fig. 4.1.

The majority of glycans are uncharged and therefore would neither be electrokinetically injected nor migrate in an electric field when EOF is suppressed. Additionally, glycans lack a chromophore/fluorophore necessary for optical detection (Guttman et al. 1996b). Thus, an ideal label for C(G)E-LIF needs to have high fluorescent yield, carry (sufficient) negative charges stable over a broad pH range (for electric field-mediated mobilization), and it needs to have an excitation wavelength corresponding to the output wavelength of commercially available lasers (commonly argon-ion laser) (Briggs et al. 2009). All these requirements are met in 8-aminopyrene-1,3,6-trisulfonic-acid (*APTS*) fluorescent dye (Evangelista et al. 1995; Ruhaak et al. 2010b), which is almost universally employed in CE-based glycan analysis today. High absorptivity and quantum yield of APTS enable a highly sensitive LIF detection, thus making CE an appropriate method for high-sensitivity glycan analysis, even with low sample amounts (Guttman et al. 1996a).

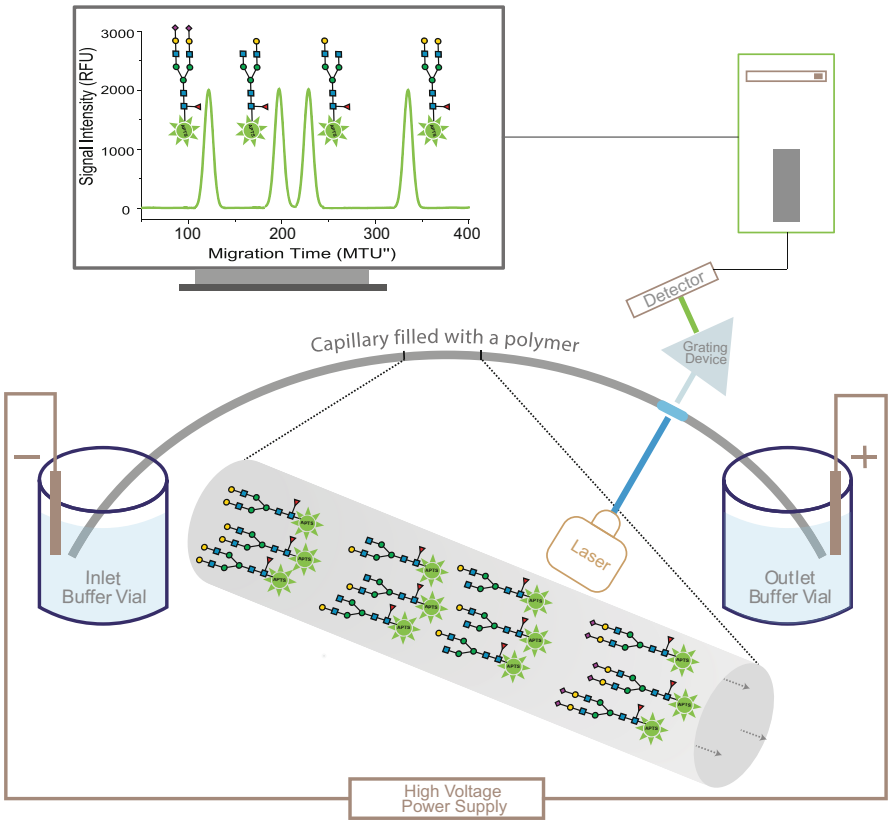

Fig. 4.1 Common separation of glycans utilizing an xCGE-LIF-based DNA sequencing system. High injection voltage is applied for a brief period, causing sample (glycans labeled with a negatively charged fluorescent dye, e.g., APTS) to enter the inlet of the gel-filled capillary by electromigration (electrokinetic injection). The electric field is applied under reverse polarity (cathode at injection and anode on the detection side) so that anions (i.e., negatively charged labeled glycans) migrate towards the detection window. As demonstrated, fluorescently labeled glycans are separated, based not only on the differences in their mass-to-charge ratios but also on their hydrodynamic volumes. Although sequencing systems benefit from multi-capillary format, only one capillary is presented here for simplicity. The x-axis of the schematic electropherogram is given in double aligned migration time units (MTU″). The signal intensity of the y-axis is given in relative fluorescent units (RFU). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

4.3 Performance, Benefits, and Potentials of Capillary (Gel) Electrophoresis C(G)E

CE provides remarkable separation efficiencies and resolution in a very short time, which makes this technology appealing for the analysis of structurally complex and diverse molecules like glycans. Recent work has shown that CE can separate even

challenging positional and linkage isomers in one single analysis run. For example, the method is capable of distinguishing even carbohydrate position in a glycan structure [e.g., nonreducing terminal residue on α1–3 arm from terminal residue on α1–6 arm of the core structure (see Fig. 4.2b, c) (Hennig et al. 2016; Schwedler et al. 2014a, b; Chen et al. 2017; Huang et al. 2017; Guttman et al. 2015)], along with position and linkage of fucose (*Fuc*) [e.g., α1–6 core Fuc from α1–3/1–4 Fuc on antenna *N*-acetylglucosamine (*GlcNAc*) or α1–2 Fuc on galactose (Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016; Weiz et al. 2016; Schwedler et al. 2014a)], type and linkage of sialic acids (*Sia*) [e.g., *N*-acetylneuraminic acid (*Neu5Ac*) from *N*-glycolylneuraminic acid (*Neu5Gc*), see Fig. 4.3d (Abeln et al. 2017), and α2–3 from α2–6 (Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016; Donczo et al. 2017; Meininger et al. 2016)], and linkage of galactose (*Gal*) [e.g., β1–3 Gal from β1–4 Gal (Konze et al. 2017; Thiesler et al. 2016; Schwedler et al. 2014a; Muñoz et al. 2019)]. This feature becomes especially advantageous when thinking about the importance of determining immunogenic α-Gal and Neu5Gc on glycoprotein therapeutics (Chung et al. 2008; Teranishi et al. 2002; Van der Linden et al. 2000). Additionally, the fact that only α2–6, and not α2–3 sialic acid, affects the anti-inflammatory activity of IgG antibody (Anthony et al. 2008) makes it crucial to have a method capable of their differentiation.

Besides speed, resolution, sensitivity, and simplicity, compatibility with a wide range of samples is one of the biggest advantages of C(G)E. Due to the robustness of the method, only a modest sample purification is necessary. Large amounts of cell debris (Konze et al. 2017; Thiesler et al. 2016; Abeln et al. 2017) or other impurities like large excess of fluorescent labeling dye (Croset et al. 2012) will not prevent a successful analysis. For comparison, in LC-based analytics, such cell debris can block the column or at least accumulate inside of it, resulting in a significant decrease of separation power. Consequently, a costly column exchange or a time-consuming column cleaning procedure is needed. In contrast, in CGE, the separation matrix (polymeric gel) is exchanged after each run; thus impure samples are not problematic in terms of clogging or sample carryover. Even sample clean-up after exoglycosidase digest—for LC an obligatory step—can be omitted, further reducing analysis time and workload (Szigeti and Guttman 2017).

Moreover, CE analysis is beneficial when enormous differences in analyte concentrations are present in one sample. For example, it could be shown by Kottler et al. that lactose, which represents up to 85% of the free oligosaccharides in human milk, did not interfere with the analysis of other more complex but low abundant human milk oligosaccharides (*HMOS*) (Coppa et al. 1993). While in xCGE-LIF the lactose peak appears without any peak shape deformation, even when injected in amounts far above the linear detection range, for LC-based methods, lactose needs to be depleted from the milk samples before HMOS analysis to avoid massive peak broadening and tailing as a result of column overload (Kottler et al. 2013). Similar problems might be faced when using mass spectrometry (*MS*).

After reviewing all the given advantages, an important aspect of the *N*-glycan analysis is the costs. Resources in academia and industry are often quite limited, especially if hundreds or thousands of samples need to be analyzed in a short time—

i.e., in real high-throughput. Here glycan analysis by xCGE-LIF, utilizing DNA analyzers originally built for Sanger sequencing, outperforms conventional LC and MS approaches. With low material costs (due to minimal sample preparation) and low operating costs (low maintenance, no organic solvents and solvent disposal costs), the overall cost are significantly lower for xCGE-LIF (Huffman et al. 2014; Mahan et al. 2015) than for LC or LC–MS. Moreover, data analysis and interpretation became user-friendly with available software solutions (see section "Data Analysis and Interpretation"), further reducing expenses for hands-on time and expertise.

Finally, C(G)E-based methods were also successfully applied for the absolute quantification of carbohydrates (Sarkozy et al. 2021; Eussen et al. 2021), which completes the wide field of applications. Taken together, unprecedented separation together with low costs, robustness, speed of analysis, multiplexing capability, high sensitivity, and nano- to femtoliter injection volumes make CE a veritable competitor to other more traditionally used techniques for glycan analysis.

4.4 Data Analysis and Interpretation

One way to obtain structural information and assign glycans to peaks is through co-injection of glycan standards, which are commercially available and fully characterized (Guttman et al. 1996a, b; Guttman and Herrick 1996; Reusch et al. 2014). In a so-called spiking experiment, the glycan is indirectly identified by the height increase of an often perfectly Gaussian-shaped CE peak. Unfortunately, only a very limited number of glycan standards are available on the market. A more general approach for the identification of glycans in a separation-based method with spectroscopic detection relies on comparing migration times (for CE separations) between a sample and a glycan database (*DB*). In this case, a fully characterized glycan standard (pure or inside of a complex mixture) is analyzed once, and its migration time is stored inside a glycan DB. The DB enables identification for all further analyses without the need for spiking the glycan standard again. Accordingly, only glycan structures with known migration times can be identified. However, the buildup of a glycan DB requires a very reliable CE setup and method with very stable and reproducible migration times.

A long-term stable migration time, independent from instrument, operator, and lab, in combination with a DB, comprising a broad variety of glycan structures, eases the structural annotation. Several research groups tackled this problem in a similar way. By running an accompanying oligosaccharide standard (e.g., a glucose ladder or a set of single oligosaccharides) for each sample or set of samples, the migration time can be aligned to this standard, resulting in a standardized time axis. Hence, the migration time of individual glycan peaks can be given in standardized migration time units (*MTU*). The MTU of a peak can now be used to search inside a dedicated DB to assign the corresponding glycan structure. Often a glucose ladder is used as an external alignment standard (in a separate run), resulting in a standardized migration

time axis in Glucose Units (*GU*) (Guttman et al. 1996a; Guttman and Herrick 1996; Mittermayr et al. 2013; Laroy et al. 2006; Mittermayr and Guttman 2012; Liu et al. 2007). Recently, it was shown that a co-injection of a bracketing triple internal standard (maltose, maltotriose, and maltopentadecaose; by Agilent) could negate the need for additional glucose ladder run (Jarvas et al. 2016)—at least for simple samples and regarding short term repeatability. As bigger variations of migration times in-between the bracketing standard can occur (region of interest), Hennig et al. use a patented orthogonal double alignment, combining the standard bracketing approach and the ladder approach (Hennig et al. 2015, 2016; Huffman et al. 2014), resulting in correspondingly double aligned migration time units (*MTU″*). An alignment to the bracketing standard, labeled with the same fluorescent dye as the sample, is complemented with an orthogonal alignment to a DNA base pair standard, labeled with a different dye. Both standards are co-injected with the sample and detected in separate spectral traces. The additional DNA ladder-supporting points for the alignment significantly improved the (long-term) stability of the migration times, which allowed to build up a large *N*-glycan DB with more than 400 structures (glyXbase™ by glyXera). Recently, the glycan analysis software glyXtoolCE™ (by glyXera) was developed (Hennig et al. 2011b, 2016; glyXera 2021), which automates migration time alignment together with an instant structural assignment and furthermore provides the background adjustment raw data smoothing, peak picking, integration, relative quantification and sample comparison (Hennig et al. 2011b, 2016; Behne et al. 2013). Similar logic is behind the recent approach from Feng et al. (2017); however, alignment was performed in a non-automated fashion and with only a single normalization point to an internal glycan standard. Related bioinformatics tools such as GUcal were developed that can carry out GU value calculation in an automated fashion and concomitantly search through the database (at the moment comprised of 92 structures, available at www.glycostore.org) (GU database) for structural assignment (Jarvas et al. 2015, 2018). All these developments facilitate data processing and interpretation and make CE an easy-to-use high-throughput tool.

Nevertheless, while spiking experiments and database comparison give a strong indication of the glycan structures, they do not fully confirm them. Also, alternative approaches, when applied alone, do not allow full structural elucidation of glycans. For example, with single-stage MS, glycan composition can be estimated based upon the addition of monosaccharide constituent masses. However, glycans with different structures but identical monosaccharide composition result in identical mass values and cannot be distinguished with MS. Even with tandem mass spectrometry (*MS/MS*), isobaric stereoisomers [like hexoses, galactose, and mannose (*Man*)], positional isoforms, and the different types of glycosidic linkages are difficult or impossible to determine. Here chromatographic and electrophoretic separations are advantageous with their ability to resolve closely related positional and linkage glycan isomers. Nevertheless, the structural variety of glycans is enormous, and resolution capabilities are not indefinite. Thus, what appears as a single peak often comprises a mixture of glycans (multi-structure peak). For that reason, also glycan analytical methods based on chromatographic and electrophoretic

separation need a second dimension to provide correct and complete sample structural information. Thus, they are often complemented with an additional technique, such as MS or exoglycosidase sequencing. Principles, benefits, applications, and some limitations of exoglycosidase sequencing are outlined in the section below. A description of different CE and MS coupling possibilities, together with the overview of recent CE–MS-based glycomics studies, is provided in section "Coupling Capillary Electrophoresis with Mass Spectrometry."

4.5 Exoglycosidase Sequencing of Glycans

Exoglycosidases are enzymes that cleave the terminal carbohydrate monomers on the non-reducing end of a glycan. They can be highly selective for specific monosaccharide types (e.g., Gal, Fuc, or Man), linkage orientation (α or β) or the position of the glycosidic linkage (e.g., 1–3, 1–4, or 2–3). Apparently, the specific enzymatic cleavage of monosaccharide residues serves as the most efficient approach for glycan structural elucidation in CE- and LC-based analysis because no additional equipment is needed. The conversion of a glycan by an exoglycosidase is harnessed with CE by analyzing the sample before and after the treatment. Digest-induced charge, size, and shape changes can be observed in CE as a change in migration time. Depending on enzyme specificity, information on monosaccharide type and the number of cleaved residues, sequence, or even linkage and anomericity can be obtained, as detailed shown by Cajic et al. (Thiesler et al. 2016) (in the supplemental material). Analysis of peak positions and relative peak areas before and after an exoglycosidase digest is therefore a means of exhaustive structural annotation. These digests can be conducted following four different strategies. First option is sequencing in a parallel fashion (Hennig et al. 2016; Thiesler et al. 2016; Feng et al. 2017), with the sample being evenly split into one aliquot per enzyme and simultaneous analysis of enzyme-treated aliquots. Second option is sequencing with parallel exoglycosidase treatments, mediated by (different) carefully designed mixtures of exoglycosidases (Szigeti and Guttman 2017; Guttman and Ulfelder 1997; Guttman 1997). Through multiple combinations of exoglycosidases (parallel enzyme array), glycans are sequenced down to their Man3-core structure (Prime and Merry 1998; Rudd and Dwek 1997). Instead of splitting the sample, it is possible to apply individual enzymes (third option) or mixtures of enzymes (fourth option) sequentially to the same sample, followed by an analysis of each step (Szigeti and Guttman 2017; Guttman et al. 2015; Mechref et al. 2005; Ma and Nashabeh 1999). This sequential treatment of a single sample is often performed when the sample amount is limited or when the sample complexity is too high. It is common practice, e.g., to reduce sample complexity by sialidase treatment before peaks are annotated (Muñoz et al. 2019; Vanderschaeghe et al. 2010; Zhuang et al. 2007, 2011). Each strategy has its advantages and drawbacks, parts of which have been already addressed elsewhere (Mittermayr et al. 2013; Prime and Merry 1998; Holland et al. 2017) and are consequently not discussed here.

Approaches to enzymatically elucidate a glycan structure in a glycan mixture are quite diverse. For instance, it was shown that the reaction of a sequential enzyme treatment could be performed inside the CE autosampler, with the sample being injected from the enzyme reaction vial directly into CE (Szigeti and Guttman 2017). Another interesting alternative to offline methods is the incorporation of enzymes into the capillary (online digest). In-capillary enzymatic digests are a rapid option, with incubation times down to only a few minutes. By passing an enzyme plug inside the capillary, the digest of the samples is accomplished during the separation process itself (mixing via polarity switching, stopped flow or low flow incubations) (Luo et al. 2010; Holland et al. 2017; Archer-Hartmann et al. 2011a, b; Yagi et al. 2011; Gattu et al. 2017; Yamagami et al. 2017). The thermally tunable phospholipid nanogels are especially attractive due to the reported enhancement of the stability and performance of exoglycosidase enzymes (Holland et al. 2017; Yamagami et al. 2017). However, in-capillary endeavors are sometimes incompatible with certain enzymes (Yagi et al. 2011; Yamagami et al. 2017) and often accompanied by a loss in separation efficiency (Archer-Hartmann et al. 2011a, b).

Although exoglycosidases can be combined with many other methods, due to already praised advantageous features of CE, combining the specificity of exoglycosidase enzymes with the strengths of CE seems most appropriate to determine glycan structures unambiguously and with minimum effort. Because of the high resolving power of CE, multi-structure sequencing of a complex glycan pool can be performed requiring no prior isolation of the individual glycans, resulting in significant labor and time savings. Even if enzymes are cleaving terminal residues without (much) specificity regarding linkage or position, CE can still provide this information due to its high separation efficiency. Thanks to the very good reproducibility of the peak areas, even small differences in glycan abundance can be detected reliably so that a digest-induced shift of a minor structure under a peak (less than 5% of total peak area) can be successfully tracked. Because of the electrokinetic loading system and highly sensitive LIF detection, only a very low amount of sample is required per injection, which means that most of the sample remains for other analyses. High-throughput capabilities obtained from multi-capillary CE formats and fast separations qualify CE as an ideal screening method of, for example, optimal reaction conditions or batch-to-batch variations. Excellent migration time stability of some CE methods allows migration time-database matching for original and exoglycosidase digested products, making analysis much faster and more efficient. All these characteristics make CE in conjunction with exoglycosidase sequencing a valuable tool for detailed glycan analysis.

Despite all acknowledged benefits, the use of enzymes for determining the glycan structure requires great care and expertise and should involve a large dose of healthy skepticism about any deduced structure (Jacob and Scudder 1994). Even if certain sugar residues are exposed at the reducing end of the glycan, these residues are not always removed by the exoglycosidases with the corresponding specificities. This resistance can be due to the strict linkage specificities of the exoglycosidases, due to steric hindrance of neighboring sugar residues or due to the attached (fluorescent) label. For example, hydrolysis of bisecting GlcNAc in human IgG *N*-glycans upon

hexosaminidase treatment or the core Fuc upon bovine kidney α-fucosidase treatment might be incomplete or completely impeded (Mittermayr et al. 2013; Laroy et al. 2006; Guttman and Ulfelder 1997; Jacob and Scudder 1994; Kamerling and Gerwig 2007; O'Flaherty et al. 2017). In addition, used enzymes may have additional selectivity for other structural features, such as local and nonlocal branching. For example, β-galactosidase can hydrolyze β1–3,4,6-linked Gal exposed at the non-reducing end of an antenna without Fuc attached to the subterminal GlcNAc. If Fuc is attached to the antenna GlcNAc, the β-galactosidase will not hydrolyze the Gal (Yu et al. 2011). Hence, a particular exoglycosidase will cleave a terminal monosaccharide only if all its specificity requirements are met. However, the purity and composition of both glycan material and used reagents can greatly affect enzyme activity and pose an additional difficulty for accurate and reliable structural assignment (Jacob and Scudder 1994). For example, variation in enzyme activity has been observed between different batches from one vendor and different sources (vendors) of the same enzyme. Enzyme activities and side activities are often tested only on artificial p-nitrophenyl glycosides or other simple substrates, even though activity on natural complex glycans can significantly differ (Kobata 2013). Additionally, vendor-added salts or other additives have the potential to interfere with different aspects of the analysis. For instance, electrokinetic injection in CE is sensitive to high salt content originating from the non-volatile digestion media (Mittermayr et al. 2013; Laroy et al. 2006), which is usually unavoidable with commercial enzymes. Finally, a major issue can be the purity of the exoglycosidase. Many of the enzyme preparations contain a certain amount of contaminant enzymes, ranging from very low to unacceptably high (Jacob and Scudder 1994; Kamerling and Gerwig 2007). Therefore, even when a positive or negative result is obtained by digestion with exoglycosidases, it does not necessarily confirm the presence or absence of the corresponding sugar residues at the non-reducing end of glycan, respectively (Kobata and Takasaki 1992). Thus, using dedicated positive and negative controls for each enzyme reaction is unavoidable.

Despite the abovementioned considerations, it was shown that, when used cautiously, exoglycosidase can effectively elucidate even subtle changes in glycan structures, including linkage type (e.g., α2–3 versus α2–6 Sia or β1–3 versus β1–4 Gal) (Callewaert et al. 2001; Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016), anomericity (e.g., immunogenic α-Gal versus β-Gal) (Abeln et al. 2017; Szabo et al. 2012; Yagi et al. 2012) and position on glycan (e.g., core Fuc versus Fuc on antenna GlcNAc or Gal) (Konze et al. 2017; Thiesler et al. 2016; Meininger et al. 2016; Liu et al. 2007; Zhao et al. 2014)—a complexity which is often inaccessible by methods other than nuclear magnetic resonance (NMR) spectroscopy.

4.6 Coupling Capillary Electrophoresis with Mass Spectrometry

Combining CE, one of the most effective isomer separation tools, with the information-rich MS technique is a mutually advantageous and powerful alliance for in-depth glycan analysis. Two setups are widely applied: the direct linking of CE to MS, the so-called online CE–MS, or a time-separated analysis, the offline CE–MS, in serial or parallel mode. For a serial offline approach, a CE instrument can be modified to spot the eluent from the capillary directly onto a target-plate for matrix-assisted laser desorption ionization with time-of-flight MS (*MALDI-TOF–MS*) detection (Suzuki et al. 1997). Consequently, separation is done by CE and identification by MS. Furthermore, parallel offline approaches are gaining popularity, especially for MS-based glycopeptide or intact glycoprotein analysis (glycoproteomics). Here, the vast diversity of protein/peptide−glycan combinations (driven by the complexity of glycans) is dramatically increasing the computing time for the interpretation of MS spectra (Thaysen-Andersen and Packer 2014; Yang et al. 2017). For reduction of computing time, an initial global characterization of the *N*-glycome can be performed by CE. The resulting glycan list is used to search against a targeted set of defined *N*-glycan structures as variable protein/peptide modifications (Thaysen-Andersen and Packer 2014; Parker et al. 2013; Lebede et al. 2021; Pralow et al. 2021; Pioch et al. 2018). This pre-knowledge of attached glycan structures greatly reduces the search space, which in turn significantly decreases the computing time. This allows relatively fast and in-depth analysis of quite complex samples, even with demanding glycoproteomic approaches.

The major advantage of online CE–MS is that glycans are identified by both their differential migration times and their molecular masses and/or fragmentation patterns in one analysis. The most common interface used for the transfer of glycans from the liquid phase of CE to the gas phase of MS is electrospray ionization (*ESI*) since this soft ionization method allows the direct transfer of the glycans from separation capillary to MS and is rather easy to implement. A detailed description of the advantages and drawbacks of the different interfacing options is beyond the scope of this chapter and has been already provided elsewhere (Simó and Cifuentes 2005; Maxwell and Chen 2008; Zhong et al. 2014; Zhang et al. 2017), together with the comprehensive overview of CE–MS-based glycomics studies (Pioch et al. 2012; Nakano et al. 2011; Mechref and Novotny 2009; Mechref 2013; Lindenburg et al. 2015; Dotz et al. 2015).

Although the CE–MS technology was introduced already 30 years ago (Olivares et al. 1987; Smith et al. 1988a, b) and continues to advance ever since, there has been relatively little work performed on merging CE with online electrospray MS for analysis of released glycans, in particular, when compared to the hyphenated LC–MS techniques. Coupling the high resolving power of CE and structural information of MS in one system often comes at the expense of resolution, sensitivity, analysis time, reproducibility and/or robustness. Very often, the separation conditions giving the unmatched separation efficiency to CE are detrimental to MS performance and

vice versa. For example, gels and buffers often used in CE because of resolution improvement are not volatile and are not suitable for CE–ESI–MS since they often suppress the ionization of the analyte, yielding poor MS sensitivity or even clog the system. On the other hand, the choice of volatile, "MS-friendly" buffers can not only affect reproducibility and analysis time but even negatively impact separations. This necessary compromise between optimal MS performance and elevated CE separation efficiency, plus its lacking robustness, are the major reasons that CE–MS has still not been widely adopted as a routine method for glycan analysis. Another concern to be addressed is that with all the limitations (small sample loads and consequent low sample concentration, sample dilution by the sheath-liquid introduction, labeling incompatibility issues, and ionization efficiency considerations) inherent in CE–MS analysis, obtaining detailed linkage and/or positional information by MS/MS is often challenging.

Even though we cannot yet talk in terms of widespread acceptance, still some efforts to make CE–MS a more viable approach in the field are ongoing. Applications are mainly limited to the use of capillary zone electrophoresis (CZE) coupled to MS for protein characterization and glycan identification. Besides the analysis of intact glycoproteins, CZE–MS is used to characterize side-specific microheterogeneity on glycopeptide level (Pioch et al. 2012; Lindenburg et al. 2015; Dotz et al. 2015). The analysis of native or derivatized glycans is often performed on complex samples (e.g., plasma *N*-glycomes), taking advantage of the good separation performance of CE (Snyder et al. 2017; Lageveen-Kammeijer et al. 2019; Huhn et al. 2012; Jayo et al. 2012). CZE–MS-based characterization of single protein glycosylation is performed less frequently (Jayo et al. 2014) and focuses mainly on monoclonal antibodies (*mAbs*) (Pioch et al. 2012; Mechref 2013; Dotz et al. 2015; Gennaro and Salas-Solano 2008; Bunz et al. 2013a, b).

4.7 Latest Developments: Miniaturization of CE Systems—Microchip CE

Easy-to-use technologies that allow rapid and efficient screening in a compact format at the best cost are in great demand, especially in the biopharmaceutical industry. Miniaturization of the electrophoretic process onto microchips (microchip CE) holds a great promise to meet all these needs. No other technology seems better suited for miniaturization owing to two main hallmarks of CE. Firstly, sophisticated pumping systems are not necessary since the separation is driven by an applied electric field. Secondly, separation efficiency depends primarily on the strength of the electric field applied along the separation capillary and not on its length (Holland et al. 1997). Most of the commercial microchip-based systems employ relatively short separation channels (<10 cm, down to few millimeters) and modest electric field strengths (<500 V/cm). However, in practice, microchip CE systems show lower separation efficiencies compared with standard CE capillary separations,

primarily due to their shorter channel lengths. Even with this apparent disadvantage, these devices provide sufficient resolution to separate and compare all major *N*-glycans found on IgG and mAbs in 60 s or less (Vanderschaeghe et al. 2010, 2013; Smejkal et al. 2010; Primack et al. 2011; LabChip Microfluidics 2018), illustrated in Fig. 4.2a.

The misconception that complex *N*-glycan samples are not amenable to separation by microchip CE has been proven wrong by recent research work. To increase the component resolution for these microfluidic separations, longer separation channels need to be fabricated on microchips. To keep the overall dimension of a chip turns had to be integrated into the channels without introducing significant sample dispersion. With these longer separation channels (>20 cm) and higher electric field strengths (>1000 V/cm), microfluidic devices are now able to rapidly and efficiently separate *N*-glycans derived even from complex samples in less than 3 min (Zhuang et al. 2007, 2011; Snyder et al. 2016, 2017; Mitra et al. 2012, 2013, 2016). Although they have great resolving power, there is often higher variability in migration times when higher separation field strengths are applied (Mitra et al. 2012).

Figure 4.2 shows a direct comparison of separations of bovine IgG-derived *N*-glycans performed by microchip CE, xCGE-LIF equipped with 50 and 22 cm capillary-arrays, and hydrophilic interaction high-performance liquid chromatography (*HILIC–HPLC*). Miniaturization of the entire electrophoretic process resulted in a reasonably satisfactory separation of the major *N*-glycan peaks in seconds, as opposed to a much better separation for xCGE-LIF and HILIC-HPLC, but in minutes. However, for glycoproteins with a low glycan complexity, the capillary length in conventional CE and CGE systems can be further shortened without considerable loss of separation power, as shown for bovine IgG analyzed by xCGE-LIF with 50 and 22 cm capillaries (Fig. 4.2b, c). An xCGE-LIF instrument equipped with a 16-channel array with capillaries of 22 cm in length has an effective separation time of 32 s per sample (8.5 min/16 samples), which is close to 22.5 s obtained by the chip-CE instrument, but with a significantly better separation of the *N*-glycan peaks. Keeping the multiplexing aspect of xCGE-LIF in mind, with its possibility to run up to 96 samples in parallel, it becomes obvious that there is potential to further increase the throughput of microchip CE.

Thus, despite the mentioned advancements, "Lab-on-a-chip" technology—incorporating both sample preparation and analysis onto the same microfluidic devices with a minimum hands-on time and being sufficiently simple for non-experts—still remains an ultimate goal. But first, the current generation of miniaturized systems has yet to demonstrate benefits in cost and performance, compelling enough to make them seriously competitive with conventional benchtop-scale CE technology. This is reflected in the current market volatility. The reliable availability of equipment, consumables, or support can often not be guaranteed. Therefore, low-cost, reliable and highly efficient microchip CE systems are expected to encourage greater use of this technology in the glycomics field.

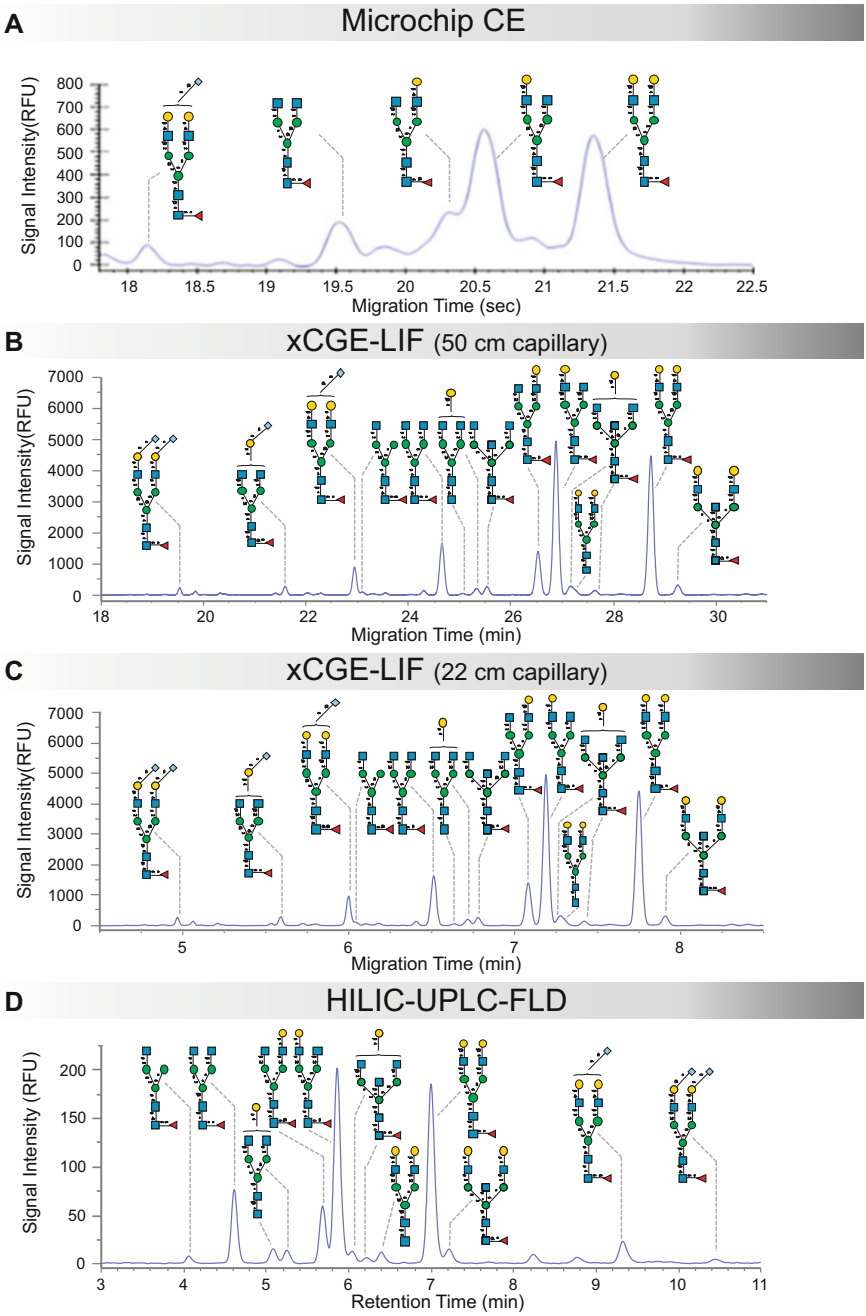

Fig. 4.2 Bovine IgG *N*-glycome analysis on the (**a**) PerkinElmer LabChip GXII Touch (microchip CE), (**b** and **c**) glyXera glyXboxCE™ built on Applied Biosystems Genetic Analyzer 3130 (xCGE-

4.8 Application of C(G)E for Immunoglobulin Analysis

Compared to common LC- and MS-based approaches, CE-based glycan analysis approaches are lagging far behind with respect to the number of applications and publications. This is largely due to the existing obstacles that needed to be tackled, including missing kits for sample preparation and non-flexible, difficult to handle or often unstable instruments. However, as discussed in detail in the previous sections, all these initial difficulties are now solved. Since stable and easy-to-handle DNA analyzers were adopted for *N*-glycan analyses by Callewaert and coworkers (Callewaert et al. 2001; Laroy et al. 2006), this approach gained popularity in the field of glycomics. Moreover, the availability of commercial analysis kits and instrumental solutions [e.g., by glyXera, PerkinElmer, Prozyme/Agilent, Sciex and Thermo Fisher Scientific (glyXera 2020; Thermo Fisher Scientific 2018; ProZyme 2018; SCIEX 2018; PerkinElmer 2018)], including software and glycan databases, made C(G)E-based methods appealing to the scientific community, as well as to the biopharmaceutical industry. This broader acceptance can be additionally attributed to the separation power of C(G)E and the ability to resolve complex glycan mixtures and to separate positional and linkage isomers. The following paragraphs are intended to highlight significant applications of C(G)E to the analyses of *N*-glycans in the science and biopharmaceutical industry and some new, exciting possibilities with a special emphasis on immunoglobulins.

IgG The C(G)E-based analysis of IgG *N*-glycosylation is well established (Reusch et al. 2014, 2015a; Routier et al. 1998) and was already applied to big cohorts (Huffman et al. 2014). Small sample quantities required for the xCGE-LIF-based analysis turned out to be quite beneficial for studies with limited sample availability, e.g., for the analysis of mouse IgG *N*-glycosylation (Patenaude et al. 2020; Schaffert et al. 2020). Consequently, this method can be applied to all kinds of mammalian samples, from large to small. As an example, Fig. 4.3 shows a comparison of the IgG *N*-glycans derived from various mammalian species, namely human, cow, mouse, dog, and rabbit. Human and bovine (cow) IgG-derived *N*-glycans show quite some similarities, despite the absence of the Neu5Gc sialic acid type in humans, respectively of Neu5Ac in cow (Raju et al. 2000). However, more dominant are the differences between the species. Their IgG *N*-glycomes vary in composition and

Fig. 4.2 (continued) LIF), (**d**) Dionex Ultimate 3000RS UPLC system equipped with Waters ACQUITY UPLC Glycan BEH Amide column. For (**a**), labeled bovine IgG *N*-glycans were prepared using the PerkinElmer Glycan Profiling Assay Reagent Kit (PerkinElmer 2018). For (**b**) and (**c**), APTS-labeled *N*-glycans were prepared from bovine IgG following a published procedure (Hennig et al. 2016; Huffman et al. 2014). For (**d**), *N*-glycans were enzymatically released from bovine IgG, AB-labeled (Ruhaak et al. 2010c) and subsequently purified (Ruhaak et al. 2008, 2012) before measurement by HILIC-UPLC-FLD. The signal intensity of the y-axis is given in relative fluorescence units (RFU). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

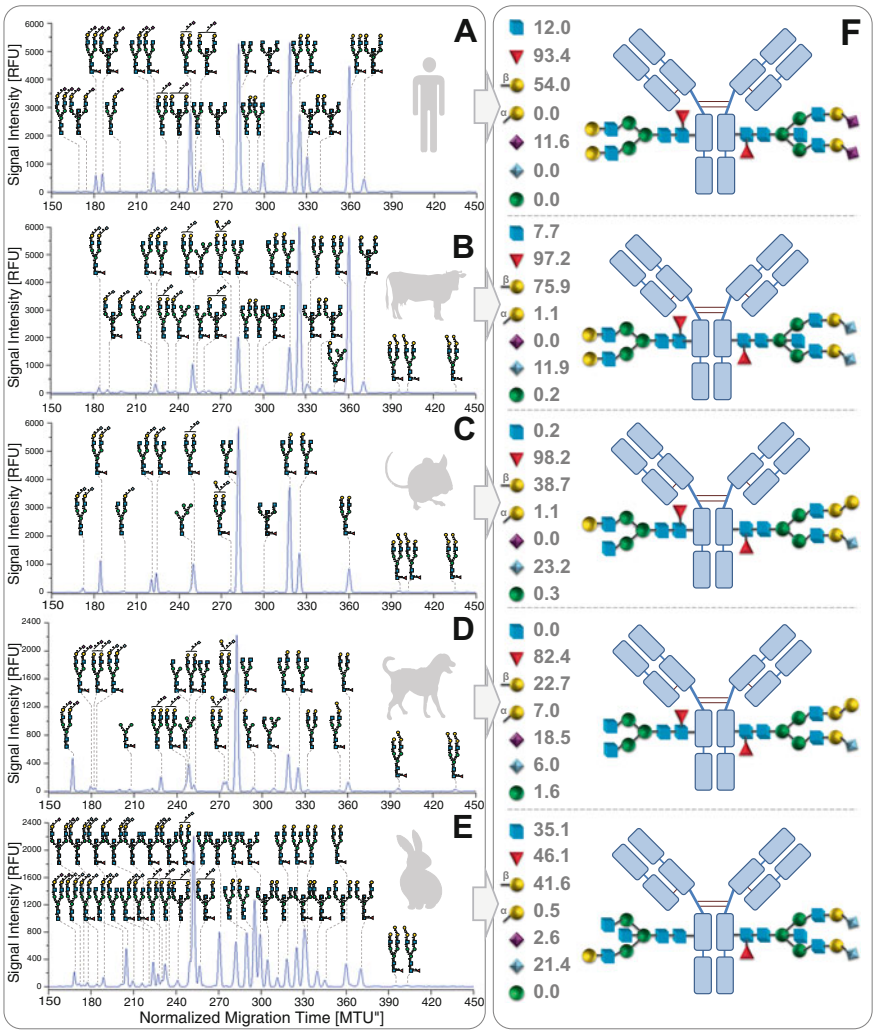

Fig. 4.3 Species-specific IgG *N*-glycosylation analyzed by xCGE-LIF. xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from human IgG (**a**), bovine IgG (**b**), mouse IgG (**c**), dog IgG (**d**) and rabbit IgG (**e**) (Vendors: Dog IgG—Abcam, other IgGs—Sigma-Aldrich). APTS-labeled *N*-glycans were prepared using the glyXprep[48] kit (glyXera 2020) by carefully following the kit instructions. Data processing was performed using glyXtoolCE™ (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU″). The signal intensity of the y-axis is given in relative fluorescence units (RFU). *N*-glycan structures were assigned via database matching using glyXtoolCE™ (in combination with glyXbase™) and confirmed by exoglycosidase sequencing as published by Thiesler et al. (2016). (**f**) Overview of IgG *N*-glycome characteristics with: blue square for bisected *N*-glycans, red triangle for core fucosylated *N*-glycans, yellow circle for glycans with terminal galactose in β1–4 or α1–3 linkage, purple diamond for glycans with terminal *N*-acetylneuraminic acid (Neu5Ac), light blue diamond for glycans with terminal *N*-glycolylneuraminic acid (Neu5Gc) and green circle for oligo-mannosidic *N*-glycans.

abundance of different glycans; from quite simple mixtures of glycans, with only a few structures (like the murine/mouse IgG in Fig. 4.3c), to extremely complex mixtures, with a wide range of structures, as shown in Fig. 4.3e for leporid (rabbit) IgG. Due to the big proportion of bisected *N*-glycans, in combination with a low degree of galactosylation and fucosylation, leporid IgG-derived *N*-glycans show one of the most complex glycosylation patterns (Taniguchi et al. 1985), which is still well resolved by xCGE-LIF, compared to results achieved by HILIC-UPLC analysis (Vainauskas et al. 2016). Additionally, the ability to separate Neu5Ac from Neu5Gc, as shown for canine (dog) IgG in Fig. 4.3d at about 180 MTU″, exposes the big potential of C(G)E-based glycan analysis techniques. As recombinant IgGs produced in different host cells is one of the major therapeutic agents to treat life-threatening diseases (Raju et al. 2000), fast and effective analysis of species-specific glycosylation differences by methods like C(G)E is becoming invaluable in this field.

Other Igs Humans have five distinct classes of Igs, namely IgA, IgD, IgE, IgG, and IgM. All Igs are comprised of two heavy and two light chains that are joined together by disulfide bonds. The glycosylation of Igs greatly varies between the different classes, ranging from only one conserved *N*-glycosylation site in IgG up to various *N*- and *O*-glycosylation sites for IgD (Maverakis et al. 2015; Clerc et al. 2016). It has long been known that aberrant IgA glycosylation causes IgA nephropathy and Henoch-Schoenlein purpura nephritis (Allen et al. 1995; Novak et al. 2007), and it was recently discovered that IgE sialylation is one regulator of allergic reactions (Shade et al. 2020). But, although Igs are the major component of the adaptive immune system, only little is known about the influence of their glycosylation, apart from the extensively studied IgG. This is partially due to current technological limitations and the complexity of their glycosylation (Maverakis et al. 2015).

Here, we show how to overcome these limitations by giving a comprehensive overview on *N*-glycosylation of all plasma immunoglobulins, analyzed by xCGE-LIF as shown in Fig. 4.4. In contrast to human IgG with incomplete sialylation (Fig. 4.4d), human IgA, IgD, IgE, and IgM show a high degree of sialylation—often in combination with a big variety of oligo-mannosidic *N*-glycan structures (Fig. 4.4a–c, e). In comparison to HILIC-HPLC results published in the early 2000s (Arnold et al. 2004, 2005; Mattu et al. 1998), the xCGE-LIF results display markedly better resolution, resolving structures appearing as multi-structure peaks in HILIC-HPLC. Guttman and coworkers achieved similar results using a CE-based

Fig. 4.3 (continued) Numbers indicate their approximated relative abundances in percent. IgG illustrations indicate the broad characteristics of the *N*-glycan structures attached to the species-specific IgGs. IgG illustrations are for visualization purposes only and do not reflect a real combination of *N*-glycans or their linkage position to the protein backbone. Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

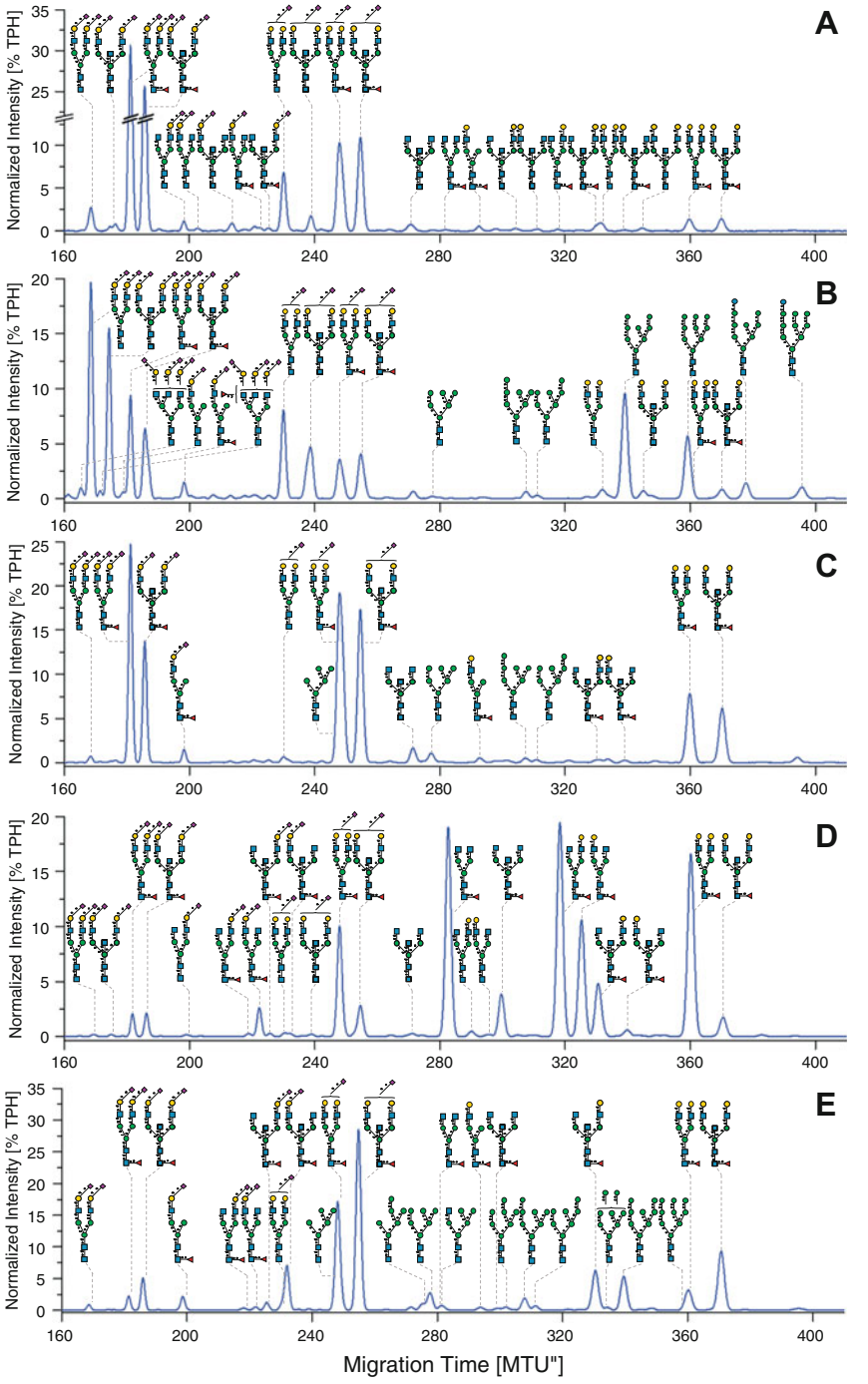

Fig. 4.4 *N*-glycan analysis of all human serum immunoglobulins by xCGE-LIF. xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from human IgA (**a**), IgD (**b**), IgE (**c**), IgG (**d**) and IgM (**e**) (Vendor: Abcam for IgA, IgD, IgE, and IgM; Sigma-Aldrich for IgG).

approach for the analysis of IgA from blood and saliva (Meszaros et al. 2020). They applied the method to biomedically relevant samples and found a link between altered IgA *N*-glycosylation and oral mucositis (Gebri et al. 2020). As the role of Ig glycosylation (besides IgG) is still not fully understood, xCGE-LIF might therefore simplify and speed up data generation and interpretation—thus, in the future promote interesting new findings when applied to big cohort studies.

Fab and Fc Antibody Domains A general approach in the field of glycobiology is to analyze released *N*-glycans of the entire IgG (the IgG *N*-glycome) (Huffman et al. 2014). However, *N*-glycans can appear at two different positions on IgG. The majority of *N*-glycans are attached to the conserved *N*-glycosylation site inside the constant (Fragment crystallizable, *Fc*) domain, while only a minor portion of *N*-glycans (up to 20%) might originate from the variable (Fragment antigen binding, *Fab*) domain of IgG (van de Bovenkamp 2019). The so-called Fab *N*-glycans differ from those attached to the Fc part, as the Fab portion possesses primarily highly sialylated and bisected complex type *N*-glycans (Clerc et al. 2016; van de Bovenkamp 2019), as shown in Fig. 4.5b (black curve), compared with the Fc-associated glycans with lower sialylation, as shown in Fig. 4.5b (blue curve). Having a global look at glycosylation of other human immunoglobulins (Fig. 4.4), the Fab glycosylation shows high similarity. The higher degree of sialylation in Fab-associated glycans of IgG might originate in part from selective removal of non-sialylated structures by the hepatic asialo-glycoprotein receptor (*HAGR*) (as for IgA, IgD, IgE, IgM, and all other blood proteins), resulting in a plasma glycoprotein typical glycosylation (Dalziel et al. 1999). Fc-associated glycans are less accessible ("hidden"), and accordingly not cleared from the bloodstream by HAGR. Furthermore, the presumably better accessibility of the IgG Fab glycosylation site to glycosyltransferases results in better processing compared to Fc glycans that are spatially localized inside the constant domain and not accessible to the same enzymes (van de Bovenkamp 2019).

Unfortunately, many big cohort studies of IgG glycosylation are focusing on released *N*-glycans and are not differentiating between IgG Fab- and Fc *N*-glycosylation (Huffman et al. 2014; Wang et al. 2017; Russell et al. 2017; Barrios et al. 2016; Trbojevic Akmacic et al. 2015; Lauc et al. 2013; Pučić et al. 2011), although recent studies suggest that the prevalence and complexity of Fab glycans might

Fig. 4.4 (continued) APTS-labeled *N*-glycans were prepared using the glyXprep^{48} kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCE™ (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU″). Peak heights were normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCE™ (in combination with glyXbase™) and confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

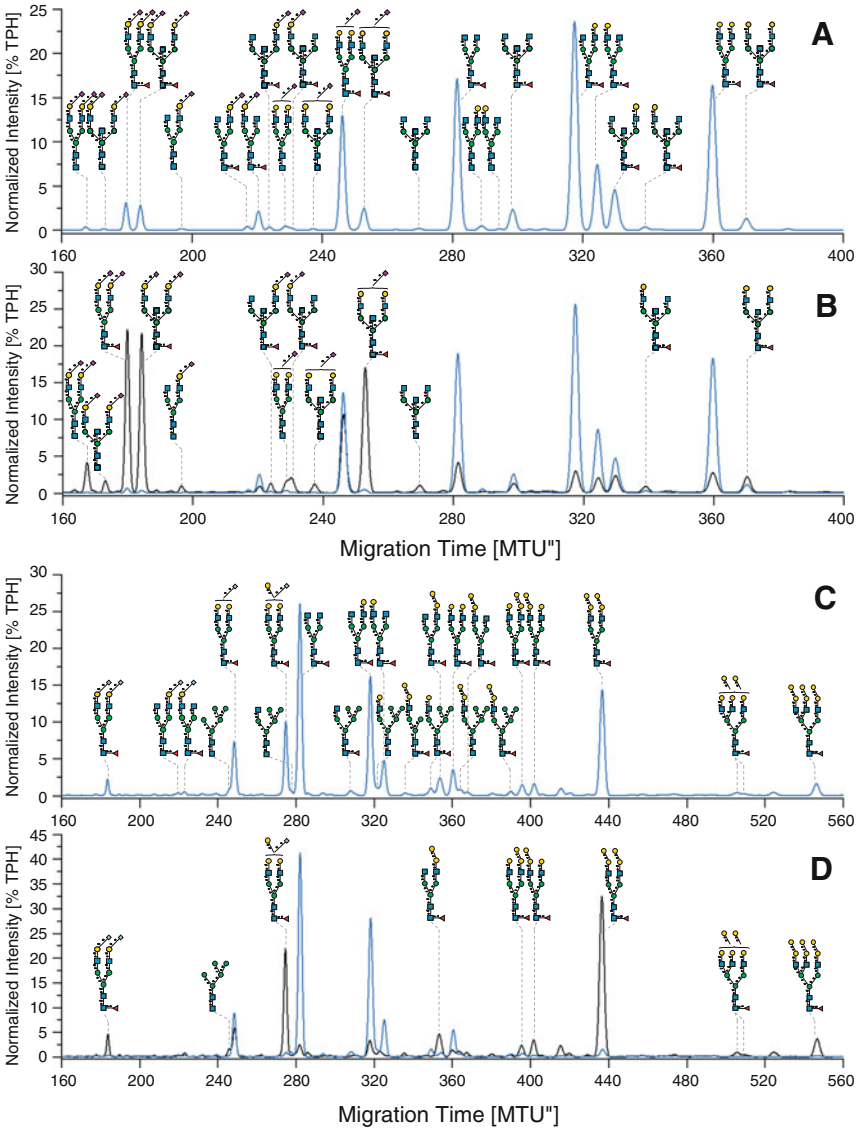

Fig. 4.5 Fab and Fc glycosylation analysis by xCGE-LIF, exemplarily shown for human IgG and the SP2/0 cell-derived mAb cetuximab (Erbitux). xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from: whole human IgG (**a**), human IgG Fab (**b**, black curve), human IgG Fc (**b**, blue curve), whole mAb cetuximab (**c**), cetuximab Fab (**d**, black curve) and cetuximab Fc (**d**, blue curve) (human IgG was purified from normal control plasma, purchased at Affinity Biologicals; cetuximab was purchased at Evidentic). Fab and Fc part of human IgG and mAb cetuximab were purified and generated as published by Bondt et al. (2014). Release and APTS-labeling of *N*-glycans were performed, using the glyXprep[48] kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCE™ (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU″). Peak heights were

change for certain physiological and pathological conditions. On the one hand, sialylated Fab glycans are presumed to be protective during pregnancy (Bondt et al. 2014), while on the other hand, an increase in Fab glycans is associated with several autoimmune diseases, like rheumatoid arthritis and Sjögren's syndrome, or cancer like multiple myeloma (Kinoshita et al. 1991). These aspects show that a separate analysis of Fab and Fc glycosylation might be beneficial for a better understanding of immunological processes and that Fab and Fc glycans might be a promising biomarker for the early detection of various diseases. Here, especially C(G)E shows big potential because of its high sensitivity—enabling detection of even the lowest amounts of Fab glycans (Fig. 4.5b), and its speed (by multiplexing)—enabling higher sample throughput if needed.

Therapeutic Glycoproteins Since glycosylation of monoclonal antibodies (mAb) is a critical quality attribute (*CQA*), the detailed characterization and control of antibody glycosylation during the development process and later product life cycle is essential. Despite the ever-growing use of C(G)E-based methods for biopharmaceutical characterization, the number of publications in the field is still quite limited (Reusch et al. 2014; Croset et al. 2012; Bunz et al. 2013a; Bielser et al. 2020; Borza et al. 2018). Nevertheless, several studies showed that the CGE-based analysis results are quite comparable to the results obtained with conventional MS- and LC-based methods (Reusch et al. 2015a, b; De Leoz et al. 2020). As shown for the SP2/0 cell-derived mAb cetuximab in Fig. 4.5c, d, the overall results are quite comparable to the earlier analysis results achieved with HILIC-HPLC and MALDI-TOF MS (Qian et al. 2007), or ESI-TOF MS (Janin-Bussat et al. 2013). Here xCGE-LIF could also resolve the isomeric structures containing the immunogenic α-Gal from non-immunogenic β-Gal for Fc, Fab (Fig. 4.5d) and complete mAb *N*-glycosylation (Fig. 4.5c). After the discovery that the α1–3 Gal epitope can cause an anaphylactic shock via an anti-oligosaccharide IgE-mediated reaction (Chung et al. 2008; Chinuki and Morita 2019), the absence of this epitope is intended for all newly developed therapeutic glycoproteins and biosimilars. Nevertheless, several previously commercialized mAbs are produced in murine hybridoma cell lines like NS0 and SP2/0, which do express the α1–3 Gal epitope (Qian et al. 2007; Uçaktürk 2012; Stadlmann et al. 2008), as shown in Fig. 4.6a for the comparison of NS0-derived mAb ramucirumab (Cyramza) (blue line) and SP2/0-derived mAb cetuximab (Erbitux) (black line). In contrast, the current dominant production cell lines are derived from Chinese Hamster Ovary (*CHO*) cells, which do not express the immunogenic α1–3 Gal epitope, as shown in Fig. 4.6b. Accordingly, the α1–3 Gal epitope can be used to identify the production cell line of a mAb in a

Fig. 4.5 (continued) normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCE™ (in combination with glyXbase™) and confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

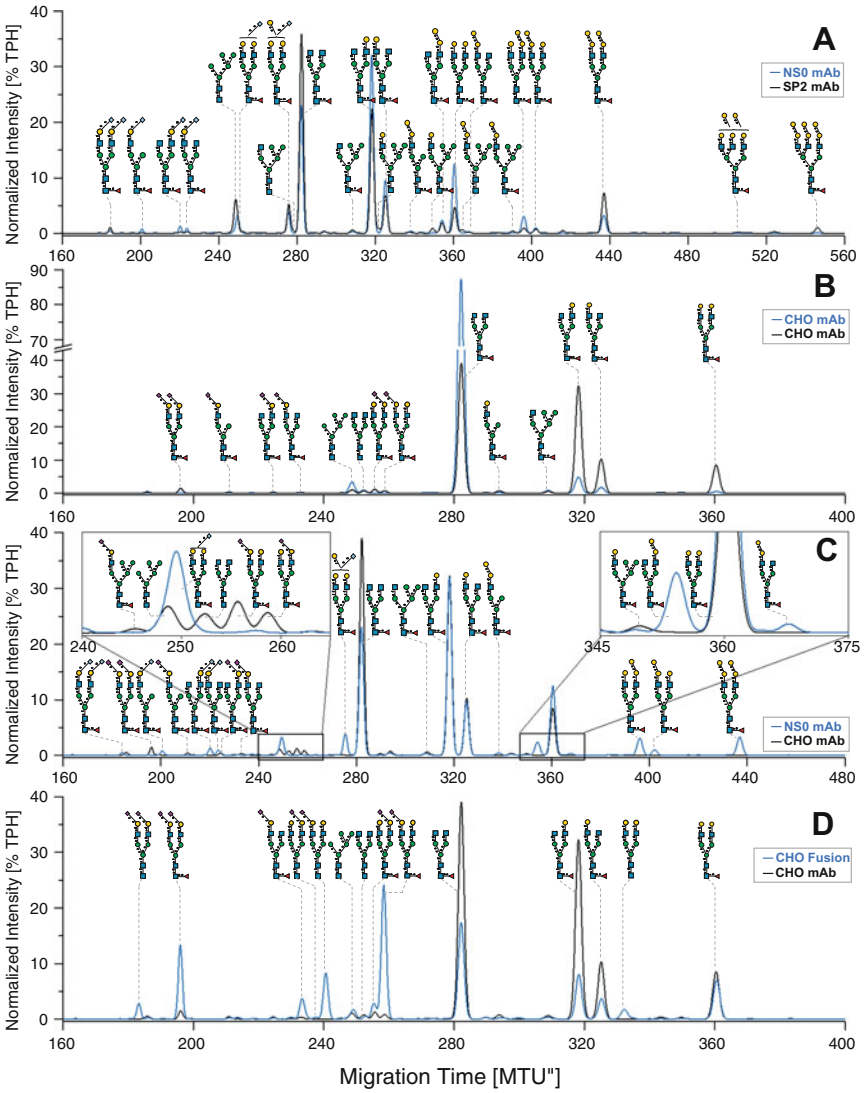

Fig. 4.6 xCGE-LIF-based *N*-glycan analysis comparing the therapeutic proteins ramucirumab (NS0), cetuximab (SP2/0), bevacizumab (CHO), rituximab (CHO) and etanercept (CHO). xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from mAb ramucirumab (Cyramza) produced in NS0 cells (**a** and **c**; blue curve), mAb cetuximab (Erbitux) produced in SP2/0 cells (**a**; black curve), mAb bevacizumab (Avastin®) produced in CHO cells (**b;** blue curve), mAb rituximab produced in CHO cells (Rituxan®) (**b**, **c** and **d**; black curve) and fusion protein etanercept (Enbrel®) produced in CHO cells (**d**; blue curve) (all purchased at Evidentic). Release and APTS-labeling of *N*-glycans were performed using the glyXprep[48] kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCE™ (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU″). Peak heights were normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCE™ (in combination with glyXbase™) and

straightforward manner, as shown for comparison of the CHO-derived mAb rituximab (Rituxan®) (black line) and the NS0 derived mAb ramucirumab (blue line) in Fig. 4.6c (zoom-in around 360 MTU″). However, CHO cells are differing from NS0 and SP2/0 cells in two additional important *N*-glycosylation properties: CHO cells incorporate mainly the non-immunogenic Neu5Ac in α2–3 linkage, as representatively shown for the mAbs bevacizumab (Avastin®) (blue line) and rituximab (black line) in Fig. 4.6b and the fusion protein etanercept (Enbrel®) (blue line) in Fig. 4.6d (Borza et al. 2018; Mcleod 2013). In contrast, NS0 and SP2/0 cells integrate Neu5Gc in α2–6 linkage (Qian et al. 2007; Stadlmann et al. 2008; Beck et al. 2008), as shown for SP2/0-derived cetuximab in Fig. 4.5c, d and ramucirumab in Fig. 4.6a. This enables an easy distinction between originator and biosimilar mAb, as both structural *N*-glycan properties (Neu5Gc versus Neu5Ac and α2–3 versus α2–6 linkage) can be directly resolved by xCGE-LIF, without the need for an additional exoglycosidase treatment, as shown in detail for the zoom-in around 250 MTU″ in Fig. 4.6c. Furthermore, the high resolution of xCGE-LIF enables the monitoring of structural *N*-glycan properties of glycoengineered CHO cells, like the missing core fucose of FUT8 knockout CHO cells (Yamane-Ohnuki et al. 2004), the additional bisected GlcNAc for GnTIII over-expression CHO cells (Umaña et al. 1999), or the emerging α2–6-linked Neu5Ac in CHO cells with stable ST6GAL1 expression (Houeix and Cairns 2019).

Besides the resolution of structural properties of *N*-glycans, the relative quantities (like the degree of galactosylation or sialylation) are easily accessible, too. As demonstrated for the mAbs bevacizumab and rituximab in Fig. 4.6b, remarkable differences in *N*-glycosylation can be observed. While bevacizumab has a considerably low degree of galactosylation (with about 90% of all *N*-glycans gathered in only one peak comprising of FA2G0), rituximab shows a more complex *N*-glycosylation pattern (with di-antennary *N*-glycans bearing up to two terminal sialic acids). In the case of sialylation, both mAbs are clearly lagging behind the fusion protein etanercept (tumor necrosis factor receptor linked to the Fc portion of human IgG1) with a sialylation degree of more than 50%. These differences in the *N*-glycosylation are often intentionally induced, as they affect the proper function of the protein (Kanda et al. 2007), its stability and thus the product potency and quality.

Moreover, xCGE-LIF is a powerful tool for high-throughput screening of hundreds of cell clones during early-stage discovery, as well as post-discovery characterization, with minimal material consumption and costs. Here, xCGE-LIF-based methods help to reduce the screening time, greatly accelerating the development of biosimilars, biobetters, and new biotherapeutics. In combination with commercially available enzymes like IdeZ (by New England Biolabs) or FabRICATOR® (Z) (by Genovis), "site"-specific characterization of IgG-based therapeutic proteins can be achieved, ensuring a more detailed level of protein characterization, and

Fig. 4.6 (continued) confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

preventing surprises during pharmacokinetic testing. Consequently, xCGE-LIF holds potential toward detailed analysis of mAb glycosylation in real high throughput.

4.9 Conclusion

CE has matured to the point that it can stay side by side with other more commonly used techniques for glycan analysis. Because of its unbeatable speed, resolution, sensitivity, and simplicity, the method is gaining more and more attention. Ongoing efforts to bring CE-based analysis kits and software solutions on the market (together with the increasing availability, size, and quality of glycan databases) will make the method attractive to the biopharmaceutical industry, helping to reveal the full potential of therapeutic glycoproteins like mAbs. Application of CE-based workflows for Ig analysis will enable more detailed characterization of their glycosylation and fast, automated high-throughput monitoring of their glycosylation patterns, especially when employed for large cohort studies. The recent and upcoming developments in miniaturization and analysis toolboxes show that there is an exciting future in glycan analysis for this recently revived technology.

Compliance with Ethical Standards

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Conflict of Interest Author Erdmann Rapp is the founder, CEO and CSO of glyXera GmbH. Authors René Hennig and Robert Burock are employees of glyXera GmbH. glyXera provides high-performance glycoanalytical products and services and holds several patents for xCGE-LIF based glycoanalysis. Author Samanta Cajic declares no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 5
Automation of Immunoglobulin Glycosylation Analysis

Jenifer L. Hendel, Richard A. Gardner, and Daniel I. R. Spencer

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Abstract The development of reliable, affordable, high-resolution glycomics technologies that can be used for many samples in a high-throughput manner are essential for both the optimization of glycosylation in the biopharmaceutical industry as well as for the advancement of clinical diagnostics based on glycosylation biomarkers. We will use this chapter to review the sample preparation processes that have been used on liquid-handling robots to obtain high-quality glycomics data for both biopharmaceutical and clinical antibody samples. This will focus on glycoprotein purification, followed by glycan or glycopeptide generation, derivatization and enrichment. The use of liquid-handling robots for glycomics studies on other sample types beyond antibodies will not be discussed here. We will summarize our thoughts on the current status of the field and explore the benefits and challenges associated with developing and using automated platforms for sample preparation. Finally, the

J. L. Hendel · R. A. Gardner · D. I. R. Spencer (✉)
Ludger Limited, Culham Science Centre, Abingdon, Oxfordshire, UK
e-mail: Jenifer.hendel@ludger.com; Richard.gardner@ludger.com; Daniel.spencer@ludger.com

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future outlook for the automation of glycomics will be discussed along with a projected impact on the field in general.

Keywords Automation · Robotization · Sample preparation · Glycan analysis · High-throughput strategies · Glycomics · Antibody

Abbreviations

2AA	2-aminobenzoic acid
2AB	2-aminobenzamide
2-PB	2-picoline borane
A1AT	Alpha-1-anti-trypsin
APTS	8-aminopyrene-1,3,6-trisulfonic Acid
AQC	Aminoquinoline carbamate
BOA	*O*-benzyloxyamine hydrochloride
CE	Capillary electrophoresis
CE-LIF	Capillary electrophoresis-laser-induced fluorescence
CHO	Chinese hamster ovary
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FAb	Fragment antigen-binding
GHP	GH polypro
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
Hpt	Haptoglobin
IBD	Inflammatory bowel disease
IgG/A/M	Immunoglobulin G/A/M
LC	Liquid chromatography
mAb	Monoclonal antibody
MALDI MS	Matrix assisted laser desorption ionization mass spectrometry
MALDI-TOF–MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MS	Mass spectrometry
MSn	Tandem mass spectrometry (MSn with $n = 2$ or 3)
NMWL	Nominal molecular weight limit
PBM	Protein binding membrane
PCR	Polymerase chain reaction
PNGaseF	Peptide:*N*-glycosidase F
RFMS	Rapifluor-MS
rhEPO	Recombinant human erythropoietin
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction

TFA	Trifluoroacetic acid
Trf	Transferrin
UHPLC or UPLC	Ultra-High Performance Liquid Chromatography
QbD	Quality by design

5.1 Introduction

Glycans are implicated in virtually all physiological processes (Varki 2017). Whilst there are a wide variety of important glycoproteins covering a wide range of functions, one of the main and widely studied groups are the antibodies. Antibodies are a group of bioactive glycoproteins with significance in both biology and the biopharmaceutical industry (Dalziel et al. 2014). As has been introduced in earlier chapters, antibodies are important in therapeutic development, understanding disease progression and provide opportunities for medical diagnostics. In this chapter, the emphasis will be on high-throughput processing of glycomics activities to support the study of antibodies in two specific areas, namely glycoprotein biopharmaceuticals and glycan biomarkers of disease for clinical diagnostics. In both of these areas, considerable advancement has been made owing to the vast improvement in analytical platforms and glycomics technologies (O'Flaherty et al. 2018; Yamamoto et al. 2016). These advancements have contributed to both a greater understanding and interest in the monitoring and optimization of glycosylation in biopharmaceutical realization and the incredible potential for clinical glycomics. However, this has also brought with it a new challenge; large sample cohorts. Therefore, this chapter is aimed at glycoscientists who are dealing with large sample sets and are interested in automation.

We will review the current status of automated sample handling and preparation of antibodies for glycomics analysis. For detailed information on biosynthesis, function, and application of antibody glycosylation, the other chapters of this book and the references therein are a good resource of knowledge. Firstly, we will focus on sample origin considerations and automated methods for purification of antibodies prior to glycan/glycopeptide generation. This section will be divided into the preparation of biological samples (specifically serum and plasma samples) and also the preparation of biopharmaceutical therapeutic antibodies. Secondly, we will look at automated methods for both glycan generation and derivatization and glycopeptide generation. Thirdly, we will discuss the advantages and drawbacks of implementing and using automation within glycomics laboratories for sample handling. Finally, we will finish off by looking at what the future holds for this subsection of the glycomics field. It should be noted that the automation of sample analysis, data acquisition and characterization using any of the common analytical platforms (LC, MS, and CE) as well as automated glycomics for other types of samples is out of scope for this review.

5.1.1 Biopharmaceutical Glycomics

Glycoprotein drugs span a range of structure-activity relationship classes. These encompass human-engineered versions of naturally occurring glycoproteins; hormones (e.g., Follicle-Stimulating Hormone; infertility), cytokines (e.g., erythropoietin; kidney dysfunction) and blood-clotting factors (e.g., Factor VIII; hemophilia). The largest class of glycoprotein drugs are monoclonal antibodies (mAbs), and the majority of approved mAbs are of the IgG1 isotype—these IgG mAbs target serious inflammatory conditions, cancers, autoimmune, cardiovascular, and infectious diseases. mAb sales are expected to reach US$130–200 billion in 2022; this is motivated by several components of the market, which include a healthy pipeline, the increasing roles for biosimilars, and also emerging economies (Grilo and Mantalaris 2019).

For mAb drugs, glycans are the greatest source of within-batch and batch-to-batch variability. Drug glycosylation impacts clinical performance (safety and efficacy), manufacturability and cost per dose. Glycosylation patterns can influence both pharmacodynamics and pharmacokinetics. Furthermore, the presence of various glycosylation features, including alpha 1–3 linked galactose and *N*-glycolylneuraminic acid can lead to adverse immunogenic reactions if they are present in biologic therapeutics. It is for these many reasons that regulatory authorities mandate that glycosylation is properly designed, measured, and controlled throughout the entire drug lifecycle. In many cases, certain glycosylation features are indicated as critical quality attributes required for therapeutic release (Costa et al. 2014).

To remain competitive, companies are seeking to optimize biomanufacturing and develop new technologies for faster drug discovery, including high-throughput screening, selection of the best clones and culturing in miniaturized bioreactor systems. Regardless of which biomanufacturing pathway is pursued, the distribution of glycan structures present on mAbs can vary. Bioprocessing parameters including; cell line, dissolved oxygen, nutrients in the feed and bioreactor type have all been shown (Costa et al. 2014; Shubhakar et al. 2016; Hossler et al. 2009) to impact glycosylation profiles, and as a result, these need to be monitored characterized and understood to satisfy regulatory scrutiny.

Striving to identify the most beneficial glycosylation patterns for drugs will lead to better and safer therapeutics but will also lead to an increase in the need for additional sample analysis. The biopharmaceutical industries' focus on obtaining optimal glycosylation in every stage during the drug lifecycle is creating a significant demand for high-throughput analysis of large sample sets.

5.1.2 Clinical Glycomics

Most proteins are glycosylated, largely as a result of post-translational modification, and these glycans play a vital role in the regulation of key biological processes, including brain development, immunity, and growth. Antibodies, which are glycoproteins belonging to the immunoglobulin superfamily, are mainly produced by plasma cells, and changes in their glycosylation profiles have been studied in response to both normal physiological processes like aging (Bonté et al. 2018) and the onset of various diseases. Indeed, abnormal glycosylation has been found as a hallmark of many human diseases like cancer, neurodegenerative, and inflammatory diseases (Dube and Bertozzi 2005; Adamczyk et al. 2012; Dennis et al. 1999). Considering this, the potential for using glycosylation signatures in biological fluids, such as plasma/serum from blood, as disease biomarkers or as a diagnostic tool for patient stratification and precision medicine is steadily becoming more attractive (Liu et al. 2019; Peng et al. 2018). The knowledge of these altered glycosylation features in plasma immunoglobulins is already leading to a greater understanding of disease pathways. However, it is yet to be determined how these features will be exploited for clinical purposes. It is possible that a new generation of clinical diagnostics could be established or alternatively, that serum glycoanalysis could be an orthogonal method to support a diagnosis by more traditional approaches (e.g. genomics).

However, to successfully implement glycan markers in clinical diagnosis, validation studies on large biological cohorts need to be performed (Shipman et al. 2020). Serum glycan profiles from different cohorts of individuals (e.g., healthy versus disease samples) need to be compared on statistically significant sample sets to identify changes in the abundance of individual glycan species accurately.

Thus, in order for glycan biomarkers to be used in clinical prognosis and diagnosis of many diseases in the future, there needs to be an analytical strategy available to characterize glycosylation on a larger-scale. However, even with the expansion of commercially available glycan analysis kits, most of the glycan biomarkers discovered so far have been studied using glycoanalytical technologies that would not be suitable for use in routine clinical diagnostic labs. The methods are limited in sample throughput, resolution, and affordability. These problems highlight the need for robust, reliable, and high-throughput sample processing methods for glycomics studies.

5.1.3 Towards High-Throughput Glycomics

Given the information discussed above, the development of reliable, affordable, high-resolution glycomics technologies which can be used for processing many samples in a high-throughput fashion is essential for both the optimization of

glycosylation in the biopharmaceutical industry as well as for the advancement of clinical diagnostics based on glycosylation biomarkers.

The status quo for glycomics studies is largely manual techniques geared towards low-throughput sample handling, often still requiring specialized expertise. In spite of the significant improvements in various chromatographic and mass spectrometric methods, the most challenging aspect remains the tedious and lengthy sample preparation steps. The most common strategies for antibody glycosylation analysis are usually either (1) enzymatic or chemical glycan release followed by chemical derivatization and measurement of the glycans by mass spectrometry, capillary electrophoresis or liquid chromatography with fluorescence detection; (2) proteolytic cleavage of the glycoprotein to produce glycopeptides which are measured by various mass spectrometry methods (Yamamoto et al. 2016).

Regardless of which of the above methods are used for glycoanalysis, additional multi-step purification processes are needed to obtain samples that will provide clear and consistent data. This means that purification is often needed to obtain a suitably pure antibody glycoprotein before using enzymatic treatment to provide glycans or glycopeptides, and further clean-up and enrichment are often needed before analysis. These technical issues make it more challenging to achieve high-throughput glycomics.

In addition to the technical issues, another challenge in glycomics is that multiple orthogonal analysis methods are often needed for the complete detailed characterization of all glycan species in a particular sample; a standard protocol may include the combination of various techniques (commonly LC, MS, and exoglycosidase sequencing) to have confidence in the structural assignments. Since characterization and analysis are so demanding, it would be ideal if the sample preparation was also not as arduous.

In an attempt to improve sample preparation and processing time, a number of high-throughput manual methods have been reported for the glycoanalysis of antibody glycoproteins from both clinical and biopharmaceutical sources (Bondt et al. 2014, 2016; Wang et al. 2016; Kumpel et al. 2020; Shajahan et al. 2019; Royle et al. 2008; Trbojević Akmačić et al. 2015). A common feature among each of these methods is that they all leverage the 96-well microtiter plate format, which has been a major enabler in the lab when handling liquid samples. In these studies, the standard low-throughput protocol for glycan analysis is often adapted to the plate format with little modification to the overall method itself, and multichannel pipettes are used to ease the amount of repetitive manual labor for the analyst.

If required, manual high-throughput purification of the antibodies is achieved by using 96-well filter plates functionalized with or containing beads functionalized with selective capture agents such as protein A or protein G.

For *N*-glycan analysis, many of the traditional methods show compatibility with processing in 96-well plates. For example, Royle et al. developed an in-gel method adapted to a 96-well plate for PNGaseF release, which was followed by 2-AB labeling and clean-up before HPLC analysis (Royle et al. 2008). This technique was first used to analyze serum IgG from a rheumatoid arthritis patient cohort and continues to be widely used for manual high-throughput antibody glycosylation

analysis applications [selected citations (Wang et al. 2016; Kumpel et al. 2020; Adamczyk et al. 2014)]. Alternatively, Bondt et al. developed a manual high-throughput method for studying FAb glycosylation at the level of released glycans obtained from serum-derived polyclonal IgG using affinity capturing beads and enzymes in 96-well plates (Bondt et al. 2014). The released glycans were subjected to ethyl esterification derivatization, resulting in linkage-specific modification of sialic acids, followed by HILIC sample purification with cotton-packed pipette tips and analysis on MALDI-TOF–MS. In addition, the focus has been given to specific parts of the process while developing manual methods for high-throughput sample preparation. For example, Trbojević Akmačić et al. found that the clean-up of labeled glycans was the biggest source of variation. As a result, they tested multiple high-throughput clean-up approaches (cellulose, silica gel, BioGel, and a hydrophilic GHP filter) for solid-phase extraction. All stationary phases were suitable for efficient purification of labeled glycans, but the GHP filter plate proved the easiest to handle and provided the most reproducible data (Trbojević Akmačić et al. 2015).

Some manual high-throughput methods have been developed with the intention of being more automation ready by slight modifications to the original methods. Shajahan et al. reported small changes to the traditional permethylation procedure for *N*-glycan derivatization in plasma, cell lines and purified glycoproteins to make the process more automation friendly (Shajahan et al. 2019). In this case, the micropermethylation reaction was conducted in a 96-deep-well polypropylene plate, and the permethylated glycans were purified by C18 tips mounted on a multichannel pipette before being analyzed by MALDI–MS and ESI–MSn. The major change to this protocol was the introduction of the C18 tip clean-up instead of the more traditional liquid-liquid extraction technique that is often used.

Likewise, Aich et al. reported on what they have called an integrated solution-based procedure for the analysis of *N*-glycans from therapeutic monoclonal antibodies (Aich et al. 2016). Their focus was to limit the number of purification, concentration, and manipulation steps to minimize the time required for sample processing and to also employ the non-toxic reducing reagent 2-PB. Therefore, all reactions starting from denaturing of proteins to the 2-AA labeling reactions were performed in the same 96-well deep plate. After completion of all reactions, the samples were purified once using a HILIC–SPE 96-well microplate using a positive pressure manifold.

Lastly, an ultrafast method for antibody glycopeptide analysis was developed by Yang et al. (2016). Here the generation and purification of tryptic glycopeptides are performed in an aqueous buffer followed by label-free quantification using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The assay time is less than 15 min, and the authors indicate that it is automation ready because the process has been designed for 96-well PCR plates using a multichannel pipette for minimal sample handling. This method was evaluated for glycoprofiling of mAbs expressed under various cell culture conditions, as well as for the evaluation of antibody culture clones and various production batches. The innovation in this method was the optimization of the trypsin digest; by employing a short digestion

time with a high temperature (70 °C) denaturing step in the presence of urea prior to trypsin addition, the generation of glycopeptides was complete in less than 10 min.

From the examples given, it is clear that the development of manual high-throughput methods for glycomics facilitates the study of large sample cohorts; however, performing manual sample preparation and manipulation is still a time-consuming process that requires significant hands-on labor. Additionally, whilst the use of manual methods is suitable for small sample sets, scaling up to larger sizes increases the likelihood of operator error leading to inconsistency and poor data repeatability. These challenges have led to increasing demand for the automation of glycomics workflows; laboratories are looking to automation as the ultimate solution for simple and scalable sample preparation methods in order to generate data that is both repeatable and reliable while also reducing the laboratory burden on their analysts.

5.1.4 Robotics: The Ultimate High-Throughput Solution?

Multipurpose liquid-handling robotic workstations have been designed to do much of the sampling, mixing, and combining of liquid samples automatically. Biology research labs and drug development labs are among the types of laboratories that have implemented these tools as solutions to limiting sample contamination and freeing up personnel to do other tasks (Alexovič et al. 2020; Kong et al. 2012).

Robotic workstations are used in glycomics studies to perform unattended actions including transport of objects (glassware, plates, racks etc.), aspiration and injection of liquids, mixing and extraction. The deck can also be expanded to include other equipment, including a centrifuge, vacuum manifolds, storage devices and incubators. As mentioned previously, the benefit of using these workstations is that the actions are all completed in a uniform fashion. When introducing an automated system into a laboratory setting, one should consider the volume that the robot is capable of handling and the corresponding precision. Other features to factor in are the footprint of the workstation (i.e., space required to accommodate the tool) and the ease of use of its software interface.

The following is a non-exhaustive list of companies who offer liquid handing robots; Agilent Technologies, Analytik Jena, Andrew Alliance, Apogent Discoveries, Beckman Coulter, Dynamic Devices, Gilson, Hamilton Company, LEAP Technologies, Opentrons, Perkin Elmer, PhyNexus, Qiagen, Tecan, and Thermo Fisher Scientific. Each of these companies offers a range of automated solutions that meet various different specifications required for each size and type of laboratory. We will not provide commentary on their comparative effectiveness or use as we are not experts on the entire range of offerings and hence would encourage the reader to seek out each individual robotics platform. We will, however, mention the robots which are used in many of the literature reports and provide the corresponding references for the readers to explore further if desired. Each lab will have a variable set-up of the robot it is using based on the equipment available and the method requirements.

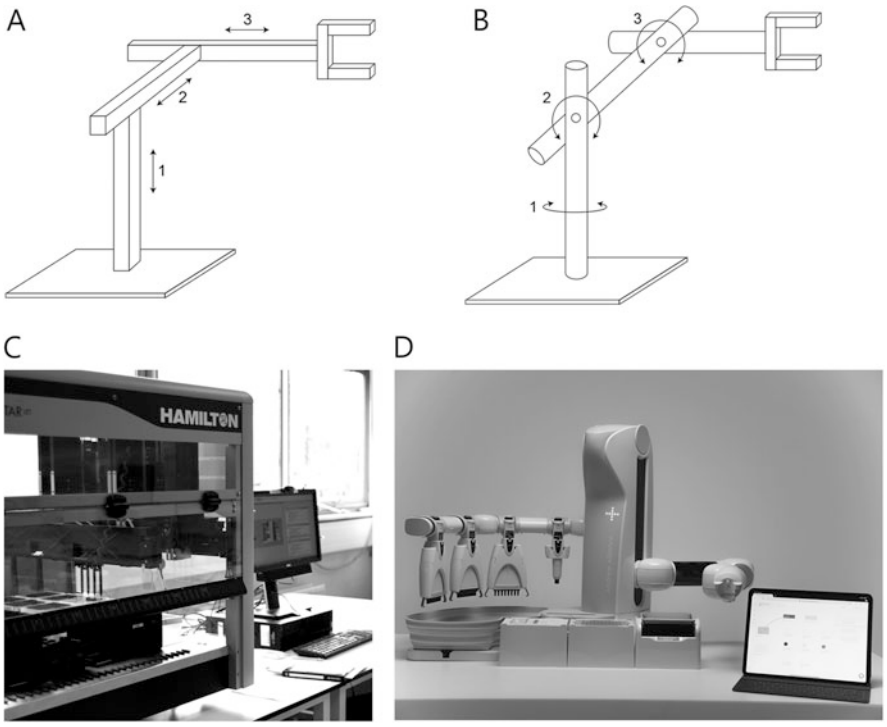

Fig. 5.1 (**a**) Representation of an anthropomorphic/articulated robotic workstation; (**b**) Representation of a Cartesian robotic workstation; (**c**) Commercial example of an anthropomorphic/articulated robotic workstation, the Andrew from Andrew Alliance; (**d**) Commercial example of a Cartesian robotic workstation, the Hamilton Microlab STARlet from Hamilton Company

The two main types of automated liquid-handling workstations which are used in glycomics laboratories fit into one of two robotic configurations: (1) the Cartesian configuration and (2) the anthropomorphic or articulated configuration (Fig. 5.1a, b respectively).

The main feature of the Cartesian robot is the programmed positioning of a portable single-probe or multi-probe injector in *x*-, *y*-, *z*-axis (Alexovič et al. 2018). The x-y-z drives control the translational motion of the end-effector, which holds the liquid-dispensing devices or plate gripper in the Cartesian coordinates. A liquid-handling robot with this structure is beneficial for its rigidity, stability and therefore, the positioning precision (repeatability), which is usually up to 10–100 microns (Kong et al. 2012). This configuration is seen, for example, in the Hamilton Star and StarLet robots, as well as the Beckmann Coulter Biomek and Tecan liquid-handling workstations (Fig. 5.1c).

The anthropomorphic/articulated robotic workstation is based on the artificial mimicking of a human arm. The core of the system represents a multi-jointed robotic arm (5- or 6-joints) with gripping fingers of various sizes at the end. Similar to Cartesian configurations, the articulated robotic arm attends to the various units

located within the working bench-area at predefined positions and levels. Here, the fingers at the end of the robotic arm are used to grip either pipettes or plastic ware (Alexovič et al. 2018). The liquid-handling robot supplied by Andrew Alliance is an example of an articulated workstation (Fig. 5.1d).

We will use this chapter to review the sample preparation processes that have been used on liquid-handling robots to obtain high-quality glycomics data of both biopharmaceutical and clinical antibody samples. This will include a focus on glycoprotein purification, glycan, or glycopeptide generation, derivatization and enrichment. The use of liquid-handling robots for glycomics studies on other sample types beyond antibodies has also been reported but will not be discussed here.

5.2 Automation of Glycomics Sample Preparation

5.2.1 Sample Origins and Protein Purification

The generation of accurate and reproducible glycomics data can only be accomplished if glycoprotein samples are free from contaminants that lead to artifacts and poor assay reproducibility (Colhoun et al. 2018); this is important for both manual and automated methods. Recent methods for glycoprotein purification exploit molecular characteristics such as solubility, size, charge, and specific binding affinity to immobilize proteins. Specific techniques include gel filtration, ion-exchange, or affinity chromatography, all of which allow the efficient removal of contaminants (buffers, reagents, and other carbohydrates and proteins) by washing the immobilized glycoproteins prior to enzymatic glycan release. However, not all options are amenable to automation for high-throughput processing. For example, although the aforementioned reproducible and robust method for glycoprotein immobilization in polyacrylamide "gel-block" (Royle et al. 2008) was used extensively in manual preparations, the gel-block hindered adaptation onto an automated platform.

Automation friendly methods for glycoprotein purification prior to enzymatic processing are divided into two categories; plasma/serum samples for biomarker and diagnostic studies and glycoprotein therapeutic antibodies.

5.2.1.1 Serum and Plasma

Glycoprofiling of the serum or plasma N-glycome is challenging due to the high complexity and heterogeneity of the glycoproteins present in these samples. Serum and plasma fractions are taken from blood samples that have undergone various biochemical protocols after collection (Tuck et al. 2010). In the case of serum, coagulation factors (i.e., fibrinogen) and blood cells are removed by centrifugation, while plasma is typically obtained from blood samples by adding an anticoagulant agent (i.e., EDTA). The glycoproteins found in serum samples are immunoglobulins

(IgG, IgM, IgA) and acute phase proteins (transferrin, alpha-1-anti-trypsin, haptoglobin). Plasma, on the other hand, contains all of the glycoproteins which are present in serum plus fibrinogen glycoprotein.

Although glycoprofiling of complete plasma or serum samples provides a comprehensive assessment of released glycans from all glycoproteins present in the liquid sample, it lacks information about the origin of the glycan and hence the identity of the carrier protein. Hence, to focus in on the glycomic information from each of the immunoglobulin antibodies, a strategy is required to selectively capture these desired glycoproteins from the mixture. This is often accomplished by affinity purification, wherein a solid surface is coated with an anti-glycoprotein-specific antibody, which is a method that has been incorporated in automated workflows. The two strategies that have been adapted for automation of antibody purification are (1) the use of 96-well affinity-protein-containing plates and (2) pipette tips packed with affinity resin.

96-Well affinity purification plates are cited most often in automated glycoprotein purification workflows. This is presumably because any plate-based tool is easily implemented onto a robot workstation deck. For the isolation of IgG or IgA from serum or plasma, plates containing either protein G or protein A are employed. These are often purchased from commercial suppliers pre-loaded with the required resin and are accompanied by documentation that clearly indicates their binding capacity. Stöckmann et al. reported the affinity purification of IgG from 20 to 50 μL of human or animal serum using a robotics-compatible 96-well filter plate containing solid-supported Protein G (Stöckmann et al. 2013). In this case, a Hamilton Robotics StarLet liquid-handling platform was used which was equipped with eight software-controlled pipettes, a vacuum manifold, and an automated heater shaker. In this example, a Thermo Scientific™ Pierce™ "Protein G Spin Plate for IgG Screening" was used, which contains 50 μL Protein G agarose resin per well capable of binding $\geq$0.5 mg of human IgG/well (ThermoFisher Scientific, n.d.). In brief, the glycoprotein is purified by binding onto the plate, washing several times with a washing buffer and then treating the plate with an elution buffer to release the purified glycoproteins. This protocol has been used to purify serum IgG from galactosemia patients (Maratha et al. 2016), Juvenile idiopathic arthritis affected children (Cheng et al. 2017) and pooled serum samples from 100 healthy male and female adults (Stöckmann et al. 2015a).

As an extension of the plate-based method discussed above, it is also feasible to combine resins to remove multiple glycoprotein antibodies of interest from serum or plasma at one time. This is a strategy that was adopted by Momčilović et al. for combined enrichment of IgG and IgA (Momčilović et al. 2020). Here, samples were prepared in 96-well plate format from minimal (~5 μL) amounts of serum using affinity purification on a Microlab STAR liquid-handling robot. Due to the difference in serum concentrations of IgG and IgA (IgG has a higher serum concentration than IgA), their simultaneous enrichment was optimized by using a mixture of IgG and IgA affinity beads in a ratio that allowed for complete capture of IgA and only partial capture of IgG. This method successfully allowed the detection and analysis

of the highly occupied glycosylation sites from both IgG and IgA in a single analytical run.

Utilizing modified pipette tips is the other automation friendly approach for affinity purification of glycoprotein antibodies. In this case, robot compatible tips are modified by packing them with the desired affinity resin for capture. Although preparation of these tips can be done manually, there are also several commercial sources of pre-packed tips for robotic platforms. O'Flaherty et al. reported the use of affinity resin-modified tips for capturing and glycoprofiling six abundant individual glycoproteins from human serum [which included the immunoglobulins, IgG, IgM, IgA, and the acute phase proteins, transferrin (Trf), alpha-1-anti-trypsin (A1AT), haptoglobin (Hpt)] by serial extraction using a Hamilton robotics StarLet liquid-handling platform (O'Flaherty et al. 2019). They used a series of both commercially available and manually packed tips containing different anti-glycoprotein capture resins (PhyNexus phytips; 20 μL of each resin). Glycoproteins from 50 μL of whole serum sample were then passed through the tips, and glycoproteins were captured in the following sequence: Trf, IgG, IgM, IgA, Hpt, and A1AT. This was optimized using a sample of pooled human serum on an automated liquid-handling station (Hamilton Starlet) in a 96-well format. This strategy proved quite successful with the captured proteins measuring >98% pure as determined by 1D-SDS page. After optimization, this method was applied to fractionate human serum glycoproteins from patients with ovarian cancer. One of the advantages of this approach is that glycomics data can be obtained for various glycoproteins from a single clinical source. The one caveat being that affinity resins with high selectivity and specificity for the targeted glycoproteins must be available.

5.2.1.2 Therapeutic Antibody Glycoproteins

The majority of therapeutic antibodies for biopharmaceuticals are produced in bioreactor-based mammalian cell cultures (e.g., Chinese hamster ovary (CHO) or murine myeloma transfectomas), and a select few are produced in other expression systems (e.g., *Escherichia coli*) (Kelley 2009). The monitoring of relevant glycan characteristics of biopharmaceuticals is required throughout the drug-life cycle, which means that these glycoproteins require testing during process development, medium development, clone selection and for final product release. To ensure reliable and reproducible glycomics data is generated, purification techniques are required to provide purified therapeutic antibodies from a variety of mixtures, some more complex than others, ranging from final formulations to fermentation broths.

In general, the same high-throughput purification strategies are applied to therapeutic glycoproteins as are applied to serum and plasma samples; the most common method used for automated therapeutic glycoprotein purification is 96-well plate-based affinity purification. For therapeutic antibodies, the trend is that 96-well affinity plates containing protein A are implemented for purification. Protein A has been chosen to exploit the strong affinity of protein A to bind to the Fc portion of IgG molecules and efficiently purify the immunoglobulin from complex mixtures such as

cell culture media. The two methods for obtaining affinity plates are to either purchase plates which already contain a specified amount of affinity resin or to prepare them in-house by adding affinity resin to standard 96-well filter plates.

Both Doherty et al. and Stöckmann et al. chose to use commercial protein A plates for purification. Doherty et al. used an automated purification method to purify IgG directly from bioreactor cell culture supernatants. IgG from the clarified medium was captured in each of the wells of a commercial Protein A filter plate (Pierce) using a Hamilton Microlab STAR liquid-handling platform. Here, glycans were successfully released from the immunoglobulin using PNGaseF while immobilized on protein A; this technique will be elaborated on further in the following section (Doherty et al. 2013). Likewise, Stöckmann et al. used preconditioned 96-well IgG affinity purification plates (Thermo Scientific, 50 μL Protein A agarose resin per well) to isolate IgG from Chinese hamster ovary cell cultures using a Hamilton Robotics StarLet liquid-handling platform. However, in contrast, they chose to elute the IgG from the resin prior to glycan processing (Stöckmann et al. 2013).

Reusch et al. opted for capturing IgG from fermentation broth by adding a protein A-Sepharose slurry (Protein A-Sepharose from GE Healthcare) to samples in a standard 96-well plate. This approach may be favorable when capturing amounts of IgG that are outside the capacity of the commercially available affinity plates. As an example of how methods are adapted to suit the capability of the automation platform, here they chose to incubate the samples and the slurry of protein A in the robot without shaking. Instead, the beads were repeatedly resuspended by pipetting up and down intermittently throughout the incubation time using a 96-channel pipetting head. The captured antibodies were eluted from the beads on a robot-mounted vacuum manifold. This procedure allowed efficient capturing and purification of IgG from a complex fermentation matrix which was confirmed by quantifying the protein content (Reusch et al. 2013).

In many of the examples mentioned, for both biological samples and biopharmaceutical samples, a cited benefit of using IgG-binding affinity resins is that they can be regenerated after use. It is important to note that this should only be recommended after a thorough validation. Tests should be done to ensure that the full binding affinity is restored after cleaning and also to check that the complete removal of all previously bound proteins is sufficient so as to not skew or contaminate the next sample set (Stöckmann et al. 2013).

5.2.2 *Preparing Glycans for Analysis: Glycan Release, Derivatization and Clean-Up*

The most common method for the investigation of glycosylation is the analysis of liberated intact glycans. As a result, the majority of automation reports describe some variation of *N*-glycan analysis. Within the realm of *N*-glycan analysis, there are several complementary multi-step analytical approaches. The workflows tend to

include the following; generally, the first step is to cleave the glycan moiety from the protein backbone using an enzymatic or chemical reaction. The gold standard method for the release of *N*-linked glycans is enzymatic treatment with peptide-*N*-glycosidase F (PNGase F); Secondly, due to the lack of inherent chromophores, it is common to derivatize the glycans with a fluorescent label after release and prior to analysis. Alternatively, the glycans can be chemically modified by permethylation or esterification to facilitate detection. Thirdly, most of these approaches require clean-up steps throughout the process, and these can be after enzymatic treatment and/or after labeling.

The following sections will detail the current literature on automated *N*-glycan sample preparation. As before, the sections will be divided by what type of sample is being processed; biological serum or plasma samples or therapeutic glycoproteins. While this helps to categorize the studies, it should be noted that it is possible for these methods to be applied across sample types, and multiple studies indicate that their processes were optimized for both.

5.2.2.1 Plasma and Serum

Automated Methods for *N*-Glycan Preparation Employing Anomeric Fluorescent Labeling Strategies

2-Aminobenzamide (2-AB) is the current gold standard fluorescent label used in glycan analysis. Therefore, it is not surprising that multiple laboratories have developed automated methods to perform this type of *N*-glycan analysis. Translation of the commonly used manual method for *N*-glycan release, 2-AB labeling and clean-up to an automated high-throughput method in a 96-well plate-based format was described by Ventham et al. (2015) In this protocol, various steps were optimized and adapted to suit the automated platform, however, the process itself closely mirrors a typical manual high-throughput method. A Hamilton Microlab STARlet liquid-handling robot was used for the automated sample processing steps, including liquid transfer and vacuum manifold mediated clean-up while all incubation steps, vacuum drying and plate sealing were carried out offline. This report is a good example of automating what is possible given the capabilities of certain laboratories. Glycoprotein denaturation, using both heat and chemical treatment, was carried out in a skirted 96 well PCR plate. A foil plate seal was employed in each of the incubation steps to ensure minimal solvent loss and to protect from contamination. To the same plate was added the PNGaseF and the respective buffers. The deglycosylation step was allowed to proceed overnight, and the solvents were removed under vacuum. *N*-glycan purification was accomplished on an automation compatible 96-well protein binding membrane plate using the integrated Hamilton vacuum manifold. The *N*-glycan solutions were transferred to a non-skirted PCR plate and dried under vacuum to enable the samples to be 2-AB labelled using reductive amination with non-toxic 2-PB reductant. A HILIC (hydrophilic liquid interaction chromatography) SPE type clean-up was implemented to remove excess

labeling reagents using LC-T1 cartridges (commercially available from Ludger Ltd.) placed into a 96-well base plate which fits onto the vacuum manifold on the robot deck. After elution from the T1 cartridges, the samples were ready for analysis by UHPLC. The repeatability of this automated *N*-glycan processing protocol was assessed by processing 48 replicates of a pooled sample of human serum IgG. The method showed excellent repeatability with a Pearson's coefficient of 0.9998 for the normalized peak areas in the IgG glycan data. This specific workflow has been used on various sample sets, which include; the study of whole serum *N*-glycosylation from IBD patients (Ventham et al. 2015), to test if different collection tubes for serum samples impact *N*-glycosylation data (Ventham et al. 2015) and also to study the whole serum *N*-glycosylation changes in pregnant women (Reiding et al. 2019).

In 2013 Stöckmann et al. reported what they called the first example of a low-cost, fully automated high-throughput assay for *N*-glycomics (Stöckmann et al. 2013).Here they used a liquid-handling robot to prepare fluorescent 2-AB labeled *N*-glycans from serum samples and indicate that the process is versatile enough to be used to determine the glycosylation pattern of individual glycoproteins or classes of glycoproteins, such as immunoglobulin G (IgG). Indeed, this protocol was used to study IgG in both biopharmaceutical samples and biological samples, including serum IgG (Stöckmann et al. 2013) as well as whole serum from healthy participants (Stöckmann et al. 2015b), plasma IgG from juvenile idiopathic arthritis patients (Cheng et al. 2017) and whole serum from pregnant women (Reiding et al. 2019). The automated process was optimized on a Hamilton Robotics StarLet liquid-handling platform and included all required steps for *N*-glycan sample preparation, affinity purification of the glycoprotein, denaturation, *N*-glycan release, fluorescent labeling and clean-up. Their strategy to optimize the automated process to ensure that all steps were completely robot compatible was elegantly described, and a few highlights of the method are; (1) They chose to use an ultrafiltration plate with a nominal molecular weight limit (NMWL) to prepare for and perform the PNGaseF digestion. This permitted the removal of excess small molecule reagents such as detergents and alkylating agents after the protein denaturation. Additionally, upon their enzymatic release from the protein, IgG *N*-glycans were easily recovered by simple filtration because their size was well below the molecular weight cut-off of the ultrafiltration membrane. (2) The released *N*-glycans were immobilized on hydrazide beads to facilitate clean-up before labeling. This was introduced because 2-AB glycan labeling after solvent removal by evaporation frequently led to inconsistent data, presumably from the presence of contaminants (buffer salts or residual detergent) interfering with the labeling reaction. To avoid time-consuming solid-phase extraction and aqueous solvent evaporation, solid-supported hydrazide beads were chosen to selectively react with reducing carbohydrates via the formation of a stable covalent hydrazone bond so that any noncarbohydrate species could be removed by a simple washing step. Once washed, the glycans could then be released from the solid support by incubation in water and catalytic amounts of acid. (3) Solid-phase extraction cartridges were used for clean-up after labeling. After the labeling reaction was quenched, to remove excess labeling reagents, the entire reaction mixture was transferred to solid-phase extraction cartridges (normal phase)

set into a 96-well plate format. Interestingly, although the glycans had been released from the hydrazide resin and are now 2-AB labeled, they found that reproducible results could only be obtained upon quantitative transfer of the entire reaction mixture, including the hydrazide resin, to the solid-phase extraction plate. Water-mediated glycan elution provided the samples, which were concentrated and ready for UPLC. The optimization of this method was successful which is reflected in its reproducibility; samples prepared on different days have coefficients of variation (CVs) that are generally below 10%. The processing time of up to 96 samples, including glycoprotein affinity purification, was around 22 h and is completely automated, built to run overnight without human intervention.

Building on the success of their previous fully automated *N*-glycan processing and 2-AB labeling platform, Stockman et al. developed an improved method (Stöckmann et al. 2015a). They chose to increase the throughput and adapted the method to a 384 well plate workflow. Additionally, they replaced the reductive amination 2-AB glycan labeling step with the "instant" aminoquinoline carbamate (AQC) labeling reaction. These changes allowed them to streamline the process significantly. The process followed the same overall workflow; glycoprotein affinity purification, glycoprotein denaturation, *N*-glycan release, and fluorescent labeling. However, multiple clean-up steps could be removed due to the nature of fluorescent labeling chemistry. In the first step, the glycoproteins were denatured and washed, and then the *N*-glycans were released from the protein using PNGase F. These steps were all carried out in the same 384-well ultrafiltration plate (10 kDa molecular weight cut-off) with vacuum filtration performed by the Hamilton Robotics StarLet liquid-handling platform. This allowed for easy washing during the denaturing step, and then the *N*-glycans could be simply separated from the protein after PNGaseF treatment. The previously automated method required *N*-glycan purification after deglycosylation, where *N*-glycans were extracted from the solution using a solid-supported hydrazide resin. In this case, residual impurities and buffer salts do not interfere with the AQC labeling reaction, hence the elimination of the need for time-consuming SPE prior to labeling. Also, no buffer exchanges were required because the deglycosylation and the subsequent AQC labeling reaction both occur at the same pH. Finally, after ACQ labeling, no final clean-up steps or solvent concentration are needed because the labeling reaction proceeds in a mixture of (30:70) buffer: acetonitrile, which is an appropriate solvent composition for injection of the sample onto the UPLC HILIC column.

It is worth mentioning that the quality of the data obtained for AQC is comparable to the 2-AB labeling method with similar *N*-glycan profiles. However, the fluorescence emission of the 2-AB profile is about 30-fold less intense in comparison to the AQC profile. This indicates that AQC labeling could be more favorable for analyzing and profiling IgG samples with a minimal amount of material. One disadvantage of the method is that migration of *O*-acetyl groups commonly found on sialic acids may occur under the conditions of release and AQC labeling. Overall, the modifications made to their previous method to develop this new fully automated AQC labeling *N*-glycan processing method had a significant impact on the throughput of their workflow, increasing the number of samples processed in a single automated

run from 96 to 768. In addition, the elimination of the SPE steps and the increased throughput significantly lowers the cost per sample, making this method an attractive one for processing large patient sample cohorts. Indeed, this technique was used by O'Flaherty et al. to perform the detailed characterization of *N*-glycosylation of six serum glycoproteins (O'Flaherty et al. 2019). They were able to study the antibodies (IgG, IgM and IgA) and acute phase proteins (Trf, Hpt, and A1AT) from a single small (50 μL) serum sample. The strength of this study and technique is that they are using one human biological fluid (serum) to obtain data on six glycoproteins in detail—this undertaking without the aid of automation would be very laborious. The utility of this method for biomarker identification in ovarian cancer was also demonstrated in the same study where the glycomics data indicates that *N*-glycosylation of Trf and Hpt glycoproteins may be suitable targets to be exploited.

As an alternative approach to the automation of a standard manual *N*-glycomics method, Nishimura et al. developed an "all-in-one" solution for automated and high-throughput *N*-glycan enrichment (Nishimura 2011). This is an integrated system wherein the selective capturing of total glycans, methyl esterification of sialic acids, and fluorescent tagging are all carried out using a hydrazide-functionalized bead handled in a multi-well filter plate. The automation of *N*-glycan release, purification, labeling, and MALDI–MS spotting were performed on the SweetBlot 7 automated system from System Instruments Co. In this study, the affinity-purified serum from 115 Ethiopian breast cancer patients and 33 healthy volunteers were studied to identify biomarkers of disease (Gebrehiwot et al. 2019). Affinity-purified IgG fractions were transferred via automated liquid-handling pipette into 96 well polymerase chain reaction (PCR) plates for denaturation, trypsin digestion and deglycosylation. At the same time, BlotGlyco H beads (Sumitomo Bakelite Co., Ltd., 10 mg/mL suspension with water) are loaded into the wells of a multi-Screen Solvinert filter plate (Millipore). The released *N*-glycan mixture is then transferred into the 96-well filter plate packed with BlotGlyco H beads. These BlotGlyco H beads are the key element in the all-in-one protocol and are stable hydrazide-functionalized polymer support. The hydrazide group on the bead reacts selectively with aldehyde or ketone groups that are present at the reducing terminus of glycans (reactive aldehyde and ketone groups are very rare in biological samples). The formation of the hydrazone bond between the BlotGlyco H bead and glycan is reversible, ensuring that the glycans can be released when needed. The unreacted hydrazide functional groups on BlotGlyco H beads are then capped by incubation with acetic anhydride. The beads are then washed to remove any impurities. This is conveniently performed in the same filter plate. The following step is on-bead methyl esterification of the sialic acid residues. This was performed to stabilize the sialylated glycans. The final steps were the transiminization with *O*-benzyloxyamine hydrochloride (BOA) fluorescent dye and mild acid hydrolysis of the hydrazone bond. The released and BOA-labeled *N*-glycans were then eluted from the filter plate under vacuum and were ready for subsequent MALDI-TOF–MS analysis. Currently, this solid-phase protocol is the only example of "all-in-one" glycoblotting technique in a single automatable workflow where all steps are completed on the bead. The automated protocol was validated by processing replicates of the same human serum

digests, and its reliability was confirmed by good repeatability (Miura et al. 2008). This all-in-one approach has been used with multiple clinical projects, including the differentiation of whole serum *N*-glycan profiles in subjects with congenital disorders of glycosylation and hepatocellular carcinoma and healthy donors (Miura et al. 2008) and the inter-ethnic differences in whole serum *N*-glycome among US origin, South Indian, Japanese, and Ethiopian ethnic populations (Gebrehiwot et al. 2018).

Automated Methods for *N*-Glycan Preparation Employing Permethylation Derivatization Strategies

The permethylation of released glycans is an alternative strategy to fluorescent labeling, and it is routinely performed prior to MALDI-TOF–MS analysis. There are a number of reasons that an analyst might choose to implement glycan permethylation, including (1) the improvement of ionization efficiency of the glycans when compared to nonderivatized oligosaccharides, (2) the stabilization of the sialic acid moieties, (3) the detection of both neutral and acidic glycans in positive ion mode on MS, (4) the ability to determine branching and glycosidic linkage positions, (5) increased glycan hydrophobicity enabling reverse-phase chromatography analysis, and (6) fast profiling and analysis times (Ciucanu 2006). However, the conventional manual permethylation techniques are laborious for large sample sets. Hence, this was the main motivation for various research groups to automate the permethylation workflow to enable high-throughput processing.

The standard permethylation method involves the following steps: *N*-glycan release, enrichment, permethylation, and liquid–liquid extraction. Shubhakar et al. were the first to adapt the majority of this method to a liquid-handling robot, specifically a Hamilton Microlab Starlet (Shubhakar et al. 2016). PNGaseF deglycosylation was performed in 96-well PCR plates with liquid handling completed on the robotic workstation. Following the enzymatic digestion, the released *N*-glycan samples were purified using a 96-well format robot compatible HILIC SPE filter plate (Ludger Clean Pre-Permethylation Clean-up Plate) followed by vacuum-mediated solvent removal. The HILIC SPE filter plate was found to provide better data after permethylation than a protein binding membrane (PBM) plate. Off-deck incubation, plate sealing and centrifugal evaporation steps were required in this process. However, liquid-handling robots are available with higher specifications which do have integrated plate sealers, incubators, and centrifuges. The use of these more sophisticated robots would allow for the total automation of the process of sample preparation and derivatization without any manual handling. The next step is the permethylation reaction. This step is carried out by dissolving the glycans in DMSO and transferring them into a 96-well plate containing solid sodium hydroxide, followed by the addition of methyl iodide to the mixture. After incubation, the permethylated glycans are isolated using liquid-liquid extraction. The most challenging aspects of this process to automate were the permethylation reaction and the liquid-liquid extraction. The liquid handling of highly volatile organic liquids, such as methyl iodide and dichloromethane which are required for these steps, is

notoriously difficult. To solve this problem, the robot was programmed to pre-wet the tips to avoid the loss of the volatile liquids and also to use the liquid level detection feature to ensure good sample recovery after four cycles of liquid-liquid extraction. One of the major benefits of using this automated procedure is that it is quick; the permethylation and liquid-liquid extraction process for 96 samples can be performed within 5 h. A second major benefit of analyzing permethylated glycans is that data acquisition using the MALDI-TOF–MS takes less than 1 min per sample. The automated permethylation method was developed and fully validated on IgG from human serum. The authors also performed a comparison study to show that the permethylated glycan data from MALDI-TOF–MS were similar to those obtained using HILIC UHPLC data and 2-AB labeling. This method has also been used to analyze other glycoprotein standards (fetuin *O*-glycans), *N*-glycan standards, and a range of biopharmaceutical samples (IgG1 mAb standard, IgG4 mAbs, and rhEPO) (Shubhakar et al. 2016).

As mentioned previously, one of the challenging steps in the standard permethylation process is the liquid−liquid extraction required for the isolation of the permethylated glycans. In addition to volatile solvents being tricky to handle, liquid-liquid extraction also comes with the risk of forming emulsions during the process as it can sometimes be difficult to obtain a clear phase separation. The process also uses known toxic solvent, dichloromethane. Additionally, liquid-liquid extraction is not suitable for recovering permethylated glycans bearing polar substituents, such as sulfates. As a way to improve the permethylation process and eliminate the need for liquid-liquid extraction, Shajahan et al. developed a high through put method which uses solid-phase extraction (SPE) to isolate the permethylated glycans instead (Shajahan et al. 2019). They chose to use this approach for the following reasons; it can be performed on smaller volumes, it does not require additional solvents, it allows for higher *N*-glycan recovery. Most significantly, SPE is easily adapted to automated workflows since it can be done in a pipet tip and C18 pipet tips are widely available from commercial suppliers.

The process follows the same steps as the standard workflow beginning with glycan release. The liberated glycans are dissolved in DMSO in a polypropylene microplate and are treated with a gel solution of sodium hydroxide in DMSO followed by iodomethane. The permethylation reaction is terminated by the addition of water which results in a bi-phasic solution due to the excess iodomethane forming a separate lower layer. In contrast to other methods where the excess iodomethane is removed during evaporation under vacuum, the iodomethane is removed by repeatedly pushing air through the mixture using a pipet to encourage evaporation directly from the plate. The final step in the process is the binding of the permethylated glycans to a C18 resin by repeatedly passing the sample solutions through C18 filled SPE tips (Thermo Fisher Scientific). The resin is washed with water, and the permethylated glycans are eluted with a small volume of methanol. The resulting solution is then ready for analysis. The full process on 96 samples from glycan release to the acquisition of MALDI-MS can be accomplished in less than 1 day. The method is very sensitive, with good *N*-glycan spectra being obtained when only 1 microgram of glycoprotein was used. This method was used to study

immunoglobulins (IgG) and transferrin isolated from human serum, along with standard glycoproteins such as bovine fetuin and κ-casein.

The authors indicate that since the entire permethylation process is carried out in conventional polypropylene 96-well plates, it can be performed manually or by using any degree of automation and that sophisticated robotics-based sample handling equipment are not necessarily required as is the case with the other automated permethylation methods. However, one caveat is that while this method has been designed for high-throughput processing in 96-well plate format and would presumably transfer to a robotic platform without issue, it is not clear that the process has been explicitly trialed and tested on an automated platform as of yet.

5.2.2.2 Therapeutic Antibody Glycoproteins

Automated Methods for *N*-Glycan Preparation Employing Anomeric Fluorescent Labeling Strategies

The biopharmaceutical industry could benefit from automated high-throughput methods designed for a variety of activities, including final product release, QC and batch-to-batch comparison. Additionally, many tasks in the development process could also be streamlined with the help of automation. These tasks include both high-throughput screening of clones for cell line selection and quality by design (QbD) studies to understand the impact of process parameters on product quality. To address the need to rapidly generate data for bioprocesses that are under development, Doherty et al. developed an automated workflow that is capable of glycosylation sample analysis directly from a bioreactor (Doherty et al. 2013). The protocol follows the standard *N*-glycan release, labeling, and clean-up strategy but with some clever optimization to reduce processing times and allow efficient automation. All steps were carried out using a Hamilton MicroLab STAR Robot, and liquid-handling steps were performed employing 8-channels, whereas sample transfer was accomplished by operating the 96-channel head. mAbs were purified from the cell culture media by capture on protein A resin in a 96-well plate format. Instead of eluting the captured mAbs from the protein A resin after washing, the glycans were removed enzymatically with PNGaseF while still immobilized. This allowed for easy isolation of the *N*-glycans without any additional clean-up steps. They were able to optimize the deglycosylation time as well, reducing the incubation time down to 60 min without a loss in yield. Complete automation of these steps was made possible by the modification of the robot deck with a custom-made integrated incubator. In this automated protocol, the reduction and alkylation steps that often precede PNGaseF release were eliminated. Not every IgG will require reduction and alkylation for efficient *N*-glycan release, but in order to see if these steps could be eliminated, they compared samples that had undergone reduction and alkylation with those that were directly treated with PNGaseF. In this case, for the samples they analyzed, there were no differences in *N*-glycan profiles. The released glycans were fluorescently labeled with 2-AB. For the final clean-up step, the excess chemical reagents were removed

using solid-phase extraction (SPE) with a bulk synthetic polyamide stationary phase (DPA-6S, 25 mg/well, Sigma–Aldrich) packed into 96-well plates and run in hydrophilic interaction mode. The advantage of this particular SPE clean-up method was that it outperformed other commercially available options, and as an added benefit, it could also be applied to the purification of underivatized glycans for complementary analytical techniques. Processing samples using this automated method takes approximately 5 h as opposed to multiple days for classical analysis and provided reproducible data for therapeutics taken directly from a bioreactor.

Although 2-AB remains the gold standard label used in the biopharmaceutical industry, an alternate fluorescent label that has been introduced recently is Rapifluor-MS (RFMS). RFMS is described as an instant label, similar to the AQC label that was mentioned previously in this chapter, that reacts "instantly" with the glycosylamine that is generated directly after PNGaseF treatment without the need of additional chemical reagents. The process of adapting the RFMS workflow to an Andrew Alliance semi-automation platform was reported by Reed et al. (2018). They used both a murine IgG1 mAb as well as the biopharmaceutical Cetuximab to optimize their automated procedure. The optimization of this protocol centered on time and temperature requirements and presented several challenges. The first challenge was that the automated platform is unable to transfer reaction vials to other locations on the robot deck. According to the manual method, sample transfer is required into and out of heating blocks set at 90 and 50 °C during the denaturation and deglycosylation steps. Here, the denaturation step requires 3 min of heating followed by 3 min at room temperature, and deglycosylation requires 5 min of heating with 3 min at room temperature. In an effort to automate this step and limit manual intervention, the robot deck was modified to include a computer-controlled Peltier effect heating block that would be used for both steps. Unfortunately, the time required to reach the desired temperatures on the heating block from room temperature was significantly longer than the required incubation times resulting in the samples being exposed to elevated temperatures for much longer than under normal conditions. This posed several problems; (1) the samples are generally uncapped and over time, evaporation becomes a concern, and (2) while the time for deglycosylation was comparable to the manual timing, the automated heating block was 10 °C off of the optimal temperature for PNGaseF digestion which can cause issues with the integrity of the glycosylamine. This was a problem because it is known that the glycosylamine produced directly following PNGase F release is not stable and converts slowly to a hemi-acetal sugar. Therefore, a prolonged delay between the enzymatic release of the glycosylamine and the labeling step will decrease the glycan labeling yield as the hemi-acetal cannot react with RFMS. In order to ensure that the manual and automated methods produced similar results, reoptimization of the denaturation and deglycosylation temperatures was performed and resulted in changing the target temperatures to 75 and 55 °C, respectively and increasing the time required for these steps. After the liberation of the *N*-glycans, RFMS labeling reagent was added to the reaction vials via a robotic pipette, which then also transferred the samples to the clean-up cartridges (GlycoWorks HILIC μElution Plate). The liquid handling is performed by the robotic arm; however, the

analyst must be present at this stage to manually manage the vacuum, turning it on and off when required. Additionally, after clean-up the waste tray must be manually replaced with sample collection tubes, and then the vacuum managed during the elution of the purified and labeled *N*-glycans.

Adapting this *N*-glycan processing strategy to an automated platform resulted in an overall increase in experiment time, with the automated protocol taking from 1 to 3 h depending on the number of samples being processed. The authors indicated that this is a significant increase in experiment time over the manually performed protocol. However, this can be justified as the manual intervention is limited to three user interactions over the whole experiment and relieves the analyst of all repetitive pipetting actions. The data obtained using the automated method was comparable to the manually performed protocol. Acknowledging the limitations of the automated method, the authors suggest this as a cost-effective benchtop solution for medium- to low-throughput laboratories. In another publication, this method was tested for its applicability in the manufacturing environment of mAb-based therapeutics by Chen et al., who used it to analyze 48 samples of infliximab derived from six different batches (Zhang et al. 2020). Based on the historical knowledge of the infliximab product, the levels of critical glycan species such as high mannose and sialylated glycans were evaluated among the samples. Indeed, using this method, they were able to identify the out-of-specification results and were able to report them with the accompanying detailed glycomics information for further investigation.

The automated method employing the AQC label (also in the instant label family), which has been described in detail for processing human IgG samples, was also used to process therapeutic glycoproteins. Stöckmann et al. applied the same automated platform for glycan release, AQC labeling and clean-up on a series of cell-culture-derived IgG *N*-glycans (Stöckmann et al. 2013). The authors were able to process antihuman IL-8 IgG successfully, showing that they could rapidly screen cell-culture-derived IgG to identify clones that produce the desired glycosylation pattern.

In many biopharmaceutical testing labs, the alternative to liquid chromatography analysis of fluorescently labeled glycans is high-performance capillary electrophoresis with laser-induced fluorescent (CE-LIF) detection. In this process, the released glycans are labelled via reductive amination with 8-aminopyrene-1,3,6-trisulfonic acid (APTS). An optimized automated method for glycan release, glycan partitioning, APTS labeling and sample clean-up for biotherapeutic mAb samples is described by Szigeti et al. (2016). In this method, the main area of innovation was the use of a magnetic bead-based protocol to avoid the centrifugation and vacuum drying steps which are often a challenge to automate. Magnetic bead-mediated techniques were first introduced in the proteomics field as a method to clean up and enrich samples using interaction-based capture mechanisms (Molares Vila et al. 2010). This technology can also be leveraged for glycans as carboxyl-coated magnetic beads can be used for the selective enrichment of glycans via solid-phase reversible immobilization. In this case, organic solvents (acetonitrile) encourage the glycans to interact with the surface of the magnetic beads allowing for the residual

peptide material left after glycan release, as well as excess ATPS reagent, to be removed from the reaction mixtures. The bound glycans can then be eluted off of the magnetic beads by treatment with aqueous media.

The fully automated *N*-glycan sample preparation platform from therapeutic glycoprotein antibodies was performed using a Biomek FXP Laboratory Automation Workstation (Beckman Coulter). The robot deck was equipped with 96-well plate holders, a magnetic stand, various pipette tip holders, solvent reservoirs, a vortex heating block and slots for sample and reagent vials. All actions were performed by the robot. The workflow for the method starts with protein denaturation and *N*-glycan release using PNGase F in a 96-well plate containing commercial magnetic beads (CleanSeq). After 1 h incubation, the *N*-glycans were captured by increasing the acetonitrile content of each well. Once the glycans were captured, the magnetic beads were held in place using the magnetic stand allowing for the removal of the supernatant. The APTS labeling of the captured glycans was then performed by reductive amination. After a 2 h incubation, the acetonitrile content of each well was increased to ensure the glycans were once again bound to the magnetic beads. The excess APTS dye was removed by washing the beads three times, and in every wash step, the following process was performed; the ATPS labeled glycans were resuspended in water, the acetonitrile content of the solution was increased to capture all glycans, and the supernatant was removed. Finally, the APTS labeled glycans were eluted from the beads by the addition of water and were ready for CE-LIF analysis.

Applying this fully automated magnetic bead-based glycan sample preparation protocol, 96 samples of commercially available murine IgG1 mAb were analyzed in less than 4 h processing time with excellent yield and good repeatability. They also showed that the method could be successfully applied to the automated sample preparation and analysis of multiple marketed therapeutic antibodies, including both the innovator and a biosimilar of adalimumab (commercial-name Humira) and trastuzumab (commercial-name Herceptin). The benefit of this method is that it is fully automated and requires no human intervention from the beginning to the end of the sample preparation process. Provided that the analytical platform of choice is CE-LIF, this method is well suited for rapid, large-scale sample processing for glycosylation analysis of biopharmaceutical antibodies.

Automated Methods for *N*-Glycan Preparation Employing Permethylation Derivatization Strategies

The automated *N*-glycan processing method using permethylation derivatization was described in Sect. 2.2.1.2 reported by Shubhakar et al. was also used to study mAbs and therapeutic glycoprotein samples (Shubhakar et al. 2016). They analyzed both a standard IgG1 mAb and also a series of IgG4 mAbs that were produced by glutamine synthetase Chinese hamster ovary (CHO) cell lines grown in stirred tank bioreactors. These cells producing IgG4 mAbs were cultured under five different bioreactor conditions varying in temperature and type of aeration. Using the

automated and high-throughput method, they were able to screen the samples from each bioreactor condition quickly and were able to identify an impact in the percentage abundance of galactosylation in the *N*-glycan profiles. This information is essential in the biopharmaceutical industry to help with clone selection and cell culture process optimization for biopharmaceutical realization.

5.2.3 Automated Methods for Glycopeptide Preparation

As mentioned in the previous section, the study of *N*-glycans is the current method of choice for glycoanalysis. However, when glycans are released from their parent antibody, the information on site-specific glycosylation is lost. On the other hand, mass spectrometric analysis of glycopeptides allows for the in-depth analysis of IgG glycosylation in a site-specific manner. These studies are required for large sample sets, especially during drug development and biomarker identification, when site-specific information is vital to the understanding and characterization of biologically relevant glycoproteins, particularly when the target antibody contains Fab as well as Fc glycosylation. The literature available on the automation of high-throughput methods for glycopeptide processing of antibodies is limited; indeed, only one publication was found that addresses this topic.

The standard method for preparing glycopeptides often includes three stages; affinity purification, tryptic digestion and clean-up. Reusch et al. chose to translate the manual method for glycopeptide analysis and adapt it to a robotic workstation (Reusch et al. 2013). This process required multiple optimization steps to make the protocol automation friendly; in this case, the biggest change was the elimination of vacuum centrifugation steps used to dry samples by evaporation. The workflow was designed on an extensively equipped Hamilton Microlab Star Robot containing: a 96-channel pipetting head and 4 single pipetting channels; grippers designed for moving labware on the robot deck; 14 cooled 96-well plate format positions for plates and troughs; 24 cooled 1.5–2.0-mL cup-holders; seven 96-well plate positions; five 96-well filter plate positions; four heated shakers; and a vacuum manifold. With this protocol, 8–384 samples could be handled in parallel (up to four 96-well plates). For the method development, they started with un-purified IgG1 and IgG4 containing fermentation supernatants and purified IgG1 in formulated bulk. The method started with the affinity purification of the glycoproteins; the authors chose to skip the vacuum centrifugation drying step that is usually directly after elution of the samples from the affinity resin. With this small change, the robotization of the entire procedure became more feasible. Trypsin was added directly to the purified IgG solutions. To increase the speed of proteolytic cleavage and to avoid incomplete digestion, two aliquots of trypsin were needed, and these were added to the reaction mixture at the beginning of the experiment and after 3 h of incubation. In addition, the 96-well plate was covered with a “robo” lid (Corning Glassware, Corning, NY, USA) to minimize evaporation during the incubation time. The purification and concentration of glycopeptides were accomplished using HILIC. Filter plates

(96-well polyethylene frit Orochem plates) were loaded with CL-4B Sepharose. This HILIC purification proved to be a crucial step for efficient glycopeptide enrichment. In order for the exclusive retention of glycopeptides on the HILIC resin, the washing buffer had to contain trifluoroacetic acid (TFA). If the TFA was not present in the buffer, both peptides and glycopeptides were detected in the mass spectra. Retained glycopeptides were eluted from the HILIC resin with water under vacuum into a 96-well collection plate. The glycopeptides were analyzed on MS directly from the elution mixture. This method for automated glycopeptide preparation showed good repeatability, and all peaks could be assigned to glycopeptide structures for IgGs produced using CHO cells. The authors were able to develop and optimize the method to allow for a high degree of automation. The optimized method was applied to measure the glycosylation state in the course of a fermentation run. Here, samples were taken from the fermentation media each day and were analyzed. They were able to establish the optimal fermentation time to obtain the desired glycosylation and could also investigate the impact of alterations in the fermentation conditions on glycosylation. The overall benefit of this method is that by analyzing glycopeptides, site-specific information on glycosylation is obtained. The authors speculated that this method would be applicable to wider glycomics applications. For example, if used with plasma samples, the method should enable the analyst to distinguish between the various IgG subtypes.

5.3 Commentary

One of the limitations of glycosylation analysis is the hands-on time required for sample preparation. As a solution to this problem, a relatively new approach that has been adopted in some laboratories is the use of automated liquid-handling robots. The status quo for the automated sample preparation of antibodies was reviewed herein. To remain in scope, only automated antibody processing for glycomics was reviewed. There are numerous reports on the automated processing of other biologically important glycoprotein samples (e.g., cells, tissues, and other bodily fluids), but they were not investigated. In general, there are a select group of researchers actively working on automated processes for glycosylation analysis workflows for antibodies. The automated preparation of antibody samples for glycosylation studies often starts with affinity purification regardless of whether the sample is a biological fluid or a therapeutic antibody. The purification of an antibody is needed to remove contaminants, other proteins or reagents, which could negatively impact the subsequent sample processing and resulting glycomic data. The affinity purification processes that have been successfully automated employ either 96-well filter plates or tips that have been packed with antibody-specific binding resins. These are either purchased from commercial suppliers or are prepared in-house. The most widely used affinity resins for glycosylated antibody purification are protein A and protein G.

The automation procedures for sample preparation of *N*-glycans, for both biopharmaceutical and biological antibodies, all use enzymatic digestion (PNGaseF) for glycan release, which is followed by a chemical derivatization step that falls into three main categories; reductive amination with a fluorescent dye, reaction with an "instant" fluorescent dye, or permethylation. Although most strategies for total automation aim to limit the total number of steps required, clean-up steps are invariably needed throughout the workflow and many groups have looked to employ different strategies to make these steps more automatable, including the use of SPE plates, immobilization of the glycans on insoluble beads and filter plates with size exclusion molecular weight cut-offs. If site-specific information about an antibody is needed, then glycopeptide analysis is the technique of choice. The automation of this process, which involves proteolytic digestion, purification, and concentration of the formed glycopeptides, has surprisingly only been reported once. In this case, the process was adapted to total automation by carrying out the proteolytic digestion in the elution mixture after affinity purification. The glycopeptides were purified and enriched using a HILIC plate and were ready for analysis upon elution.

After reviewing the literature on the feasibility and overall success of robotizing glycomics workflows for antibody glycosylation analysis, there were several trends emerged. The first is that plasticware is a pivotal item for translating manual processes to automated processes. Plates are a key point of automation friendly methods; whether they are filter plates packed with various affinity resins, clean-up resins or size exclusion membranes, the commercial availability of these products makes a huge difference in the ease of translation. Likewise, pipette tips are just as integral to the success of an automated process. Secondly, transferring a manual high-throughput procedure onto an automated platform requires a good amount of optimization and ingenuity from the analyst. The analyst must also be able to program the corresponding software and run simulation experiments to ensure that the computer and liquid-handling robot are properly "talking." Furthermore, out-of-the-box solutions are often needed to overcome roadblocks; for instance, translating heating or shaking step might demand an alteration to the method to allow automation—the analyst may ask: Do I really need to do this step? Or can it be done in a different way? Instead of shaking, will be repeated up and down pipetting result in the same outcome? Likewise, the analyst may consider if a certain type of antibody glycoprotein requires denaturation or if the numerous centrifugation and vacuum—centrifugation steps that make full automation a challenge could be eliminated. In some cases, the resolution is to automate what is possible given the equipment and resources available. Finally, it is common practice for test methods to be run in simulation mode before initiating the actual run to maximize productivity and minimize any potential errors.

There are several key advantages of using automated methods. (1) Automation eliminates most human errors resulting in more reliable data. The use of a precision device increases confidence in the accuracy of sample preparation, and this is then reflected in the quality of the resulting data. (2) Automation removes the physical stress that repetitive manual pipetting creates for scientists and also reduces the risk of exposure to hazardous materials used in certain experimental procedures.

(3) Many more samples can be processed at the same time. Automation allows for higher throughput, and many platforms can facilitate the queuing of samples for continuous or parallel processing. In addition, once a process is automated, a further increase in throughput can be achieved by converting the method to a 384-well plate format. (4) Fully automated processes mean that there is no human intervention required at any point in sample processing, allowing analysts to focus on other tasks, freeing up people resources. (5) The computerized control of the robot means that protocols can be easily transferred between labs by sharing the programming code. (6) Robots are able to provide sophisticated computational features such as; monitored air displacement pipetting, independently spreadable pipetting channels and program logs indicating errors in the process. The disposable tips can have integrated conductivity probes that allow for liquid level detection, and they can also be fitted with anti-droplet control for pipetting volatile organic solvents. In addition, when the process is semi-automated, the robot programming can prompt the analyst. For example, if a robot was not equipped with an on-deck incubator at this step, an on-screen instruction can be set to instruct the user to manually perform the incubation and to press "OK" when the required action was completed. (7) Lower costs per sample for processing. Typical consumable costs are less than the manual low-throughput equivalents. Also, the increase in precision may allow for smaller volumes of initial starting material, which translates to smaller amounts of reagents as well. Specifically, for the biopharmaceutical industry, the high-throughput screening capabilities can allow for faster data generation allowing for quicker decision making on the optimal cell processing parameters or clone selection and thereby reducing overall expenditure.

There are various challenges associated with developing and implementing robotized methods. (1) One of the biggest roadblocks is that not all aspects of sample preparation are readily automated, and the ease of translation often depends on the sophistication of the equipment. Full automation is difficult to achieve. (2) Another challenge when using a liquid-handling platform is that dead volumes must be considered in cost analysis. The solvent and reagent reservoirs must have sufficient volume for liquid transfer, and this will always mean that excess is required. For cheap reagents, this is not a concern but for expensive reagents, dead volume can lead to increased cost. (3) Sample stability and evaporation of solvents is a concern on robotic workstations—although some now come with plate sealing options or are capable of maneuvering lids—the time without this is still a potential problem. (4) The start-up costs for implementing robotics are high. Indeed, a huge limiting factor to the adaptation and expansion of automated glycomics research is that acquiring a liquid-handling robot requires a significant upfront capital investment for the machine itself and additionally, there is the cost of expert personnel for method programming and development. Therefore, a laboratory may require a high sample demand to make automation worthwhile. (5) While hands-on time will be limited, the automated process does not always offer any time savings in comparison to the manual method. (6) Robotic platforms require users who are comfortable and proficient in the programing language or operating system provided with the machine.

5.4 Future Perspectives

The future outlook for the advancement of automated methods to support both biopharmaceutical realization and medical glycomics is promising. The ever-growing demand for higher throughput studies will keep the field moving; the increasing size of sample sets coming from both biopharmaceutical and clinical sources has been a relatively recent challenge to the glycomics field, and this will inevitably drive continuous improvement and further development of automation. The biopharmaceutical pipeline is currently dominated by mAbs, and in the future, this will only increase as more innovator mAbs are developed, and new biosimilars of off-patent therapeutics are released. Likewise, there has been tremendous growth in the clinical glycomics field, with many glycosylation-based disease biomarkers being discovered. In the future, the translation of these biomarkers to clinical diagnostic tools will require high-throughput solutions for a large number of patient samples. Progress continues to be made to adapt automation to all aspects of glycomics sample preparation. Indeed, out of the publications reviewed herein regarding the automated preparation of antibodies for glycoanalysis, three were published in the first quarter of 2020. The advancement in automation equipment and capabilities will be a key factor in the continued expansion of the available automated methods for glycosylation analysis of antibodies. This will be impacted by the improvement in both robot compatible plastic ware as well as improvement in solid-phase separation, affinity and size exclusion resins and their commercialization in 96- or 384-well formats. Finally, there is hope that the cost burden of implementing automation will be driven down by both its wider use and also by the publication of literature supporting its effectiveness for high-throughput glycomics studies.

5.5 Conclusions

Antibodies are glycosylated proteins that are key molecules in two major fields; the biopharmaceutical industry and medical glycomics. Scientific progress in both of these areas has steadily increased the demand for higher throughput sample processing. In the biopharmaceutical industry, the importance of glycosylation on the efficacy and safety of mAbs, which have proven to be hugely successful for treating illness, has made glycomics monitoring vital at all stages of drug development. At the same time, significant developments showing that the identification and diagnosis of the disease can be facilitated by the study of human antibodies is driving interest in the use of clinical medical glycomics.

From the examples provided in this chapter it is apparent that automation offers a viable solution to minimize variability due to human error, provide greater consistency, and reduce the effort required for sample preparation in glycomics studies. Multiple research groups have successfully been able to optimize and adapt

glycomics protocols for both *N*-glycan and glycopeptide preparation to liquid-handling robot platforms. While there are limitations to the uptake of automated methods in many laboratories, the main advantage is that sample preparation protocols that were once extremely labor-intensive are now adapted to robotic platforms, which allow IgG *N*-glycan analysis with throughput capabilities that are much greater than before. These developments will help stimulate the interest in large-scale glycan analysis, which continues to grow and become more popular in life science as researchers realize its importance in biological processes. This growing interest should lead to high throughput glycan analysis becoming more common in academic, industrial and clinical centers and has a great potential to impact wider society, ultimately leading to an improvement in human health and well-being.

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Chapter 6
Bioinformatics in Immunoglobulin Glycosylation Analysis

Frédérique Lisacek, Kathirvel Alagesan, Catherine Hayes, Steffen Lippold, and Noortje de Haan

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F. Lisacek (✉)
Proteome Informatics Group, SIB Swiss Institute of Bioinformatics, Geneva, Switzerland

Computer Science Department, University of Geneva, Geneva, Switzerland

Section of Biology, University of Geneva, Geneva, Switzerland
e-mail: frederique.lisacek@sib.swiss

K. Alagesan
Max Planck Unit for the Science of Pathogens, Berlin, Germany

C. Hayes
Proteome Informatics Group, SIB Swiss Institute of Bioinformatics, Geneva, Switzerland

Computer Science Department, University of Geneva, Geneva, Switzerland

S. Lippold
Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

N. de Haan
Copenhagen Center for Glycomics, University of Copenhagen, Copenhagen, Denmark

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Abstract Analytical methods developed for studying immunoglobulin glycosylation rely heavily on software tailored for this purpose. Many of these tools are now used in high-throughput settings, especially for the glycomic characterization of IgG. A collection of these tools, and the databases they rely on, are presented in this chapter. Specific applications are detailed in examples of immunoglobulin glycomics and glycoproteomics data processing workflows. The results obtained in the glycoproteomics workflow are emphasized with the use of dedicated visualizing tools. These tools enable the user to highlight glycan properties and their differential expression.

Keywords Glycomics · Glycoproteomics · Workflow · Software · Database · Glycoinformatics

Abbreviations

ETD	Electron-transfer dissociation
Fc	Fragment crystallizable
HTP	High throughput
Ig	Immunoglobulin
IMGT	ImMunoGeneTics information system
LC	Liquid chromatography
MIRAGE	Minimum Information Required for A Glycomics Experiment
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	nuclear magnetic resonance
PDB	Protein Data Bank
PNGase F	Peptide-*N*-glycosidase F
RP	Reverse phase
RT	Retention time
SNFG	Symbol Nomenclature for Glycans

6.1 Introduction

Glycosylation plays a major role in creating proteoform diversity reaching the magnitude of 10^6 in the human proteome. The mapping of this diversity is far from being charted, particularly in the case of glycoforms, mainly because of the limitations and the very broad variety of experimental techniques needed to capture information. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) as part of a fuller range of analytical methods (Gray et al. 2019) are used to solve the structure of glycans. In recent years, the improved accuracy of mass spectrometers and sophisticated fragmentation techniques continue to refine the identification of

intact glycopeptides (peptide with attached glycan) and new glycoproteomics datasets are regularly published (Ye et al. 2019; Riley et al. 2019).

Glycomics and glycoproteomics have benefitted from past experience in proteomics and the principles of the Proteome Standards Initiative (Orchard et al. 2003) and ProteomeXchange (Vizcaíno et al. 2014) are being gradually applied. In the past decade, reporting guidelines have been issued through the MIRAGE (Minimum Information Required for A Glycomics Experiment) initiative (https://www.beilstein-institut.de/en/projects/mirage/) as part of the many minimum information standards defined to facilitate data checking and processing (Kolarich et al. 2013).

Determining a glycosylation profile may entail either glycomics or glycoproteomics studies or both, depending on experimental design. In most cases, mass spectrometry data analysis software is used. Despite the expansion of glycan (Fujita et al. 2020) and glycoconjugate databases and repositories (Alocci et al. 2019; York et al. 2020), direct use of such resources is limited for glycan identification as there is no template that can be used to infer structures detectable in an organism. Some of the commonly applied strategies rely on established proteomics search tools that are refined to identify multiple modifications or exploiting idiosyncratic fragmentation patterns of glycans, while others use dedicated software for glyco(proteo)mics data, as exemplified in this chapter.

Glycosylation analyses of proteins can be performed either at intact glycoprotein, at glycopeptide, or released glycan level. Irrespective of the level of analytical method, structural analysis of glycoproteins remains elusive due to lack of high-throughput analytical tools and data analysis pipeline capable of analyzing high-volume spectral data to determine the glycan composition, structure and/or glycopeptide sequence information (Pralow et al. 2020; Alagesan et al. 2020). Immunoglobulins (Igs) and Fc receptors are complex glycoproteins and key components of both the innate and adaptive immune systems. The specific glycosylation of both immunoglobulin and its receptors is well known to be crucial for maintaining and modulating effector functions. In humans, there are five distinct antibody isotypes: IgM, IgD, IgE, IgA, and IgG. All of them are glycosylated (Arnold et al. 2007; de Haan et al. 2020). IgG provides an ideal case scenario for the development of high-throughput analytical tools and data analysis pipeline as it (1) is one of the most abundant proteins in human serum, accounting for about 10–20% of plasma protein, and (2) mostly bears a single *N*-linked glycan attached to Asn-297 of each heavy chain, which can have a wide variety of different structures. These unique IgG features allow for fine-tuning and extensive benchmarking of high-throughput (HTP) glycoanalytical methods (Trbojević-Akmačić et al. 2016; Wuhrer et al. 2007; Gstöttner et al. 2020; Amez-Martín et al. 2020).

This chapter briefly surveys the landscape of glycan and intact glycopeptide identification and quantification bioinformatics resources and focuses on profiling of antibodies using glycomics or glycoproteomics approaches as well as using visualization tools to easily compare these profiles. The coverage of resources is depicted in Fig. 6.1.

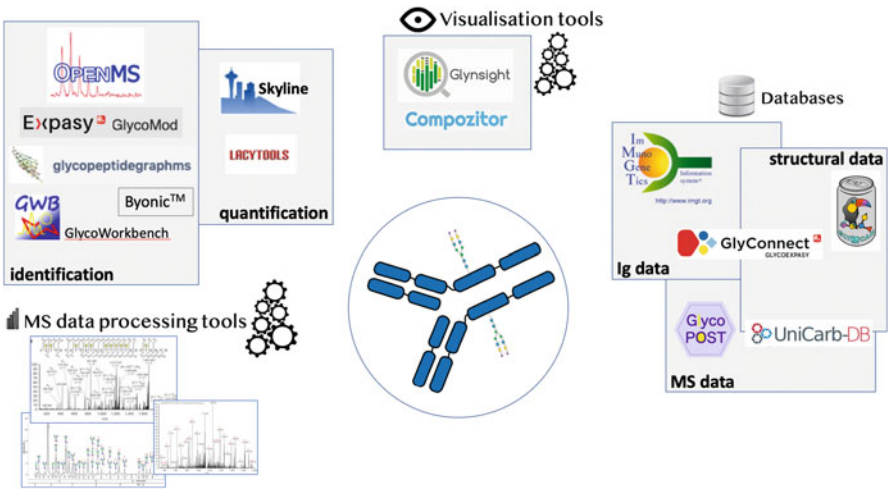

Fig. 6.1 Summary of resources. The glycoinformatics resources that are cited and used in this chapter are categorized as either database or tool. The tools are further distinguished according to their purpose: tools processing mass spectrometry (MS) data (identification or quantification) or used to visualize processed data. Databases store different types of data

6.2 Glycomic Data Collection and Processing

A recently published chapter covers widely the database and tool collection available for glycomics studies (Lisacek et al. 2017) presented as exhaustive lists of the various databases and software tools. The present section is an update on recent additions to the collection that is not reproduced here but complemented by the partially overlapping content of Table 6.1.

6.2.1 Reference Databases

In recent years, several community-driven moves in glycan bioinformatics have been initiated, the first of which is the agreement to resort to a single repository for glycan structures. This resulted in the creation of GlyTouCan in 2016, only briefly mentioned in (Lisacek et al. 2017) as version 1.0 (Aoki-Kinoshita et al. 2016). GlyTouCan is an uncurated registry for glycan structures that assigns globally unique accession numbers. It is increasingly adopted as a reference (Tiemeyer et al. 2017) and reached version 3.0 in 2021 to include a substantially reshaped registration flow improving data submission (Fujita et al. 2020). Manually annotated and experimentally verified spectra collected in UniCarb-DB (Hayes et al. 2011) are cross-referenced to GlyTouCan in order to increase the speed and accuracy of glycan assignment.

Table 6.1 Summary of cited glycoinformatics resources

Type	Resource name	Availability	References
Databases			
Glycoproteins	GlyConnect	glyconnect.expasy.org	Alocci et al. (2019)
Immunoglobulins	IMGT	imgt.org	Lefranc et al. (2015)
Glycans	UniCarb-DB	unicarb-db.expasy.org	Hayes et al. (2011)
3D structures	Protein Data Bank	www.wwpdb.org	wwPDB consortium et al. (2019)
Proteins	UniProt	uniprot.org	The UniProt Consortium (2019)
Repositories (Enabled data submission)			
MS data	GlycoPOST	glycopost.glycosmos.org	Watanabe et al. (2020)
Glycans	GlyTouCan	glytoucan.org	Fujita et al. (2020)
Software			
Generic MS Processing	ProteoWizard	proteowizard.sourceforge.net/tools.shtml	Kessner et al. (2008)
	OpenMS	www.openms.de	Pfeuffer et al. (2017)
Glycan MS Processing	GlycoMod	web.expasy.org/glycomod	Cooper et al. (2001)
	GlycoWorkbench	code.google.com/archive/p/glycoworkbench	Ceroni et al. (2008)
	GRITS toolbox	www.grits-toolbox.org	Weatherly et al. (2019)
Glycopeptide MS processing	Byonic	Licensed	Bern et al. (2012)
	GlycopeptideGraphMS	bitbucket.org/glycoaddict/glycopeptidegraphms/src/master/	Choo et al. (2019)
Quantitative MS processing	Skyline	skyline.ms/project/home/begin.view?	Pino et al. (2020)
	LacyTools	git.lumc.nl/cpm/lacytools	Jansen et al. (2016)
Visualization	Glynsight	glycoproteome.expasy.org/glynsight	Alocci et al. (2018)
	Compozitor	glyconnect.expasy.org/compozitor	Robin et al. (2020)

Glycomics mass spectrometry data have been taken one step further with the introduction of a MIRAGE-compliant experimental data repository named GlycoPOST (Watanabe et al. 2020) and the implementation of a pipeline for collecting such data (Rojas-Macias et al. 2019). Some publishers have started imposing data deposition and the field of glycoscience is slowly (re)connecting to life sciences.

6.2.2 Identification and Quantification Software Tools

GlycoMod (Cooper et al. 2001) and GlycoWorkbench (Ceroni et al. 2008) are still in use in much the same way for the identification of glycan structures, described in (Lisacek et al. 2017). These earlier protocols (1) from analytical MS data to monosaccharide composition using GlycoMod, (2) from structure to predicted MS/MS data using GlycoWorkbench, and (3) from analytical MS and MS/MS data to structure using UniCarb-DB, are still valid. A more recent identification platform is the GRITS toolbox (Weatherly et al. 2019) that can be considered as an upgrade of GlycoWorkBench. In particular, managing adducts is made more flexible and larger datasets composed of thousands of spectra are more easily handled.

Quantification was barely addressed in (Lisacek et al. 2017) mainly due to the limited number of quantitative glycomics datasets a few years back. The situation is rapidly changing and the need for related software is increasing. Skyline is accurately described by its authors as a software ecosystem for quantitative mass spectrometry informatics (Pino et al. 2020). This platform efficiently tackles issues such as large dataset management, integration with other commonly used tools, data visualization, independently of the experimental workflow. This approach made the tool popular in many -omics using mass spectrometry irrespective of the size of identified molecules, that is, ranging from metabolites to large peptides, encompassing glycans and glycopeptides. Application to serum glycoprotein site occupancy using Skyline was reported early in (Hülsmeier et al. 2016). Since then, the use of the tool has spread both in glycomics and glycoproteomics.

6.3 Glycoproteomics Data Collection and Processing

6.3.1 Reference Databases

As hinted in the introduction, glycoproteomics is fast-growing and three appropriate databases have been released in recent years. In fact, the GlycoPOST repository (Watanabe et al. 2020) was designed for mass spectrometry data submission in relation to JPOST, the Japanese member of the ProteomeXchange Consortium. A database of curated glycoproteins and their associated glycans called GlyConnect (Alocci et al. 2019) includes many entries describing glycosylated

immunoglobulins. This database is reciprocally cross-linked with UniProt (The UniProt Consortium 2019), the reference protein sequence database, and feeds glycomics data to GlyGen (York et al. 2020), the recently released US portal for glycoscience.

6.3.2 Identification Software Tools

The topic of automatic intact glycopeptide identification is regularly reviewed (Thaysen-Andersen and Packer 2014; Hu et al. 2017; Cao et al. 2020; Abrahams et al. 2020), following the abundant production of new tools that warrant frequent updates of the catalog. This chapter is not destined to add to this set of reviews.

One of the key points in running glycopeptide identification software is the selection of the glycan composition file upon which the identification of the glycan moiety is based. In the majority of cases, the composition file can be qualified as "empirical" since its definition relies on glycan data collected from the literature or from databases/repositories. It is, however, an unevenly flexible definition. In some software, it is a modifiable parameter while in others it is not. Alternatively, the knowledge of glycan biosynthesis can be used to generate expected glycans. For example, MAGIC (Lih et al. 2016) starts with 29 monosaccharides to be combined according to enzymatic rules and in a similar way, 19 groups of seed structures are defined in GlycoPAT (Liu et al. 2017). In this way, the composition file is "theoretical" as the result of potential enzymatic activity. This definition is also flexible since both the enzymes or the monosaccharides can be removed or constrained.

Most classical proteomics search engines accommodate the selection of glyco-based modifications from a collection they provide. For example, in principle, Mascot (Savitski et al. 2011) relies on the UniMod subset of glycans (Creasy and Cottrell 2004). The user may also use in-house definitions as in (Bollineni et al. 2018), where a few hundred compositions were built from a customized and systematic addition of monosaccharides prior to using the Mascot engine. Note that instrument vendors also provide a glycopeptide identification component in the software suites that are usually available for proteomics data analysis such as ProteomeDiscover by Thermo or ProteinScape at Bruker, but the information on glycan composition files is not easily accessible.

Proteomics software developers, in particular when posttranslational modifications are accounted for, have already acknowledged that the performance of search engines correlates with limiting the search space (Schwämmle et al. 2015). Similar observations have been made in glycoproteomics and this viewpoint, also promoted here, is strongly advocated in (Khatri et al. 2017).

6.3.3 *Quantification Software Tools*

Most glycopeptide identification tools have some sort of built-in quantification module, either based on a spectral count or MS1 feature intensity or integrated area. While these modules are often sufficient to obtain a rough estimation of (relative) glycopeptide abundance, more sophisticated software tools have been reported for accurate glycopeptide quantification in a high-throughput manner. As described above, Skyline is well suitable for the quantification of glycopeptide data and has excellent visualization capability that allows for data curation and representation (Pino et al. 2020). LacyTools is an automated data processing tool for high-throughput analysis of LC-MS glycoproteomics data and is suitable for the simultaneous analysis and quality control of thousands of samples (Jansen et al. 2016). Both tools feature important options such as quality control based on isotopic pattern matching, separate integration of different charge states, and a flexible inclusion of isotope signals.

6.4 Data Integration with Other Omics

In the early years of bioinformatics in the 1980s when the term itself was hardly used, the main focus was on collecting and comparing gene and protein sequences. A handful of resources created then are still available now and this is the case of the international ImMunoGeneTics information system (IMGT), created in 1989 by Marie-Paule Lefranc in Montpellier, France. This resource remains a unique gene-centric reference in immunogenetics and immunoinformatics (Lefranc et al. 2015). IMGT/3Dstructure-DB is a subsection of the IMGT platform where Ig three-dimensional information is included. This data is cross-referencing the Protein Data Bank (PDB) (wwPDB consortium et al. 2019), where glycan information is symbolically represented in protein structures using a 3D version (Sehnal and Grant 2019) of the Symbol Nomenclature for Glycans (SNFG) depiction increasingly adopted in publications and textbooks (Varki et al. 2015).

Since the PDB has long considered glycans decorating glycoproteins as "ligands," IMGT lists "glucid" as potential ligands of immunoglobulins. IMGT/3Dstructure-DB (version 4.12.3) contains 21 entries where 3D structures include information on attached glycans. Figure 6.2 summarizes the steps of the search and illustrates the possible visualization of attached glycans with the example of the PDB entry 5 K65 of VEGF binding IgG1-Fc (Fcab CT6), as recorded from (Lobner et al. 2017).

The recently introduced feature of the PDB clearly showing the presence of a glycan on the 3D representation of the protein is the first step toward bringing together different data sources that cover complementary information on immunoglobulins. Further work in this direction is needed to reach a better level of integration between genomics and glycomics.

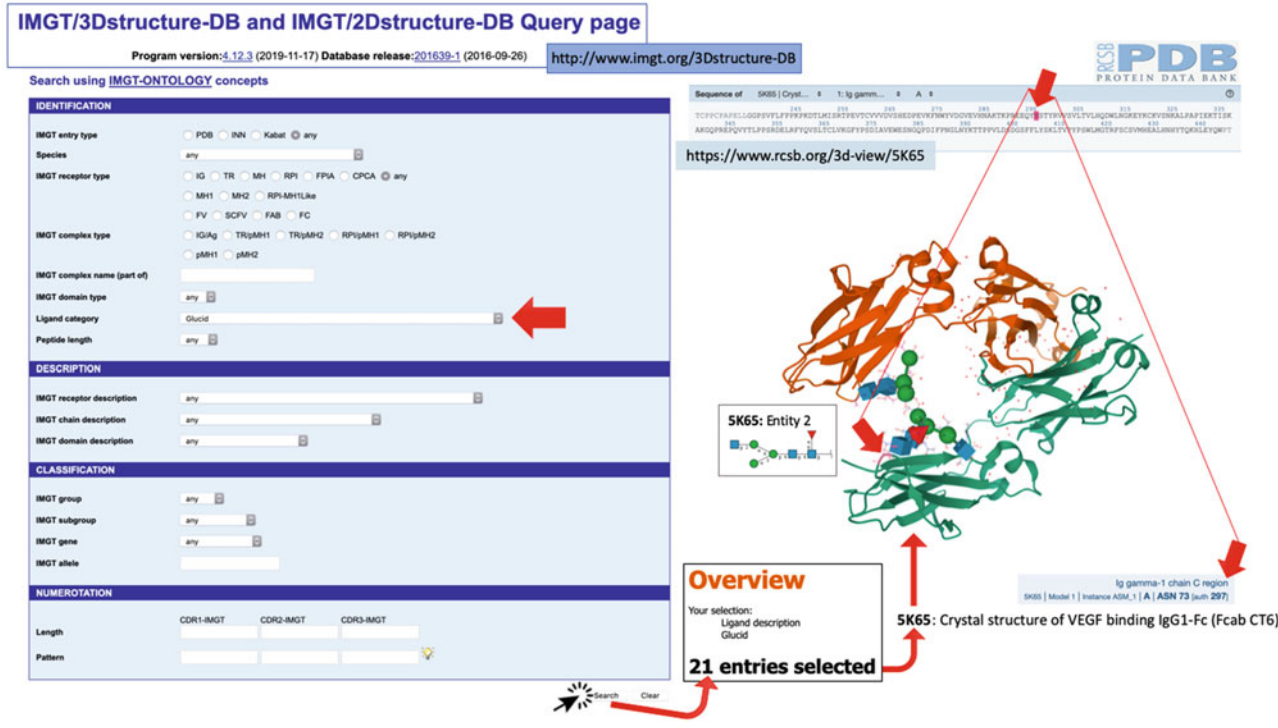

Fig. 6.2 Potential data integration with IMGT. The IMGT platform integrates genetic, structural, and functional information about immunoglobulins. It is split into several sections, one of which is devoted to 3D structures. Querying this section by ligand category with the "glucid" term (central red arrow) outputs a list of 21 entries with cross-links to the Protein Data Bank (PDB). The new interface of the PDB highlights the glycan in the 3D structure with the recommended SNFG notation

6.5 Practical Examples

This part describes the stepwise processing of glycomic and glycoproteomic Ig MS data, as well as the visualization of selected glycoproteomic identification results. Supplementary information is provided in the Notes section (notes 9–12), at the end of the chapter.

6.5.1 Glycomic Data Processing

The glycomic approach to Ig *N*-glycan characterization is traditionally performed after isolating the Ig of interest from its complex matrix by affinity chromatography. Subsequently, the *N*-glycans are released from their carrier protein using the enzyme Peptide-*N*-Glycosidase F (PNGase F). Prior to MS analysis, glycans may be subjected to permethylation, reduction, or reducing end labeling to increase sensitivity and specificity. Analysis of released glycans allows for in-depth structural elucidation and isomer separation, but it lacks information on the site of glycosylation. Therefore, for Ig-specific glycan analysis at the glycomics level, protein purity is of utmost importance. The glycomics data analysis protocol outlined below allows for the structural determination of reduced glycans released from human IgG. The workflow is illustrated in Fig. 6.3.

6.5.1.1 Software Required

1. *Manual inspection of raw data*: Bruker Daltonic Software (Bruker DALTONIK GmbH, Bremen, Germany) was used since the example data was generated with a Bruker instrument (amaZon speed Electron-transfer dissociation (ETD) ion trap mass spectrometer).
2. *Data format conversion*: MSConvertGUI (ProteoWizard 3.0 suite, http://proteowizard.sourceforge.net/)
3. *Determination of glycan composition from the experimentally determined precursor mass*: GlycoMod (https://web.expasy.org/glycomod/) that works well for both free and derivatized glycan as well as for glycopeptides.
4. *Interpretation of tandem MS spectra* (MS/MS spectra): GlycoWorkbench (https://code.google.com/archive/p/glycoworkbench/)
5. *Quantification*: Skyline (64-Bit) 20.2.0.343 (https://skyline.ms/project/home/begin.view?)

6.5.1.2 Glycan Composition Determination

1. Determine the experimental glycan masses. Convert any doubly and triply charged m/z values to singly charged or neutral mass. Bruker Data Analysis

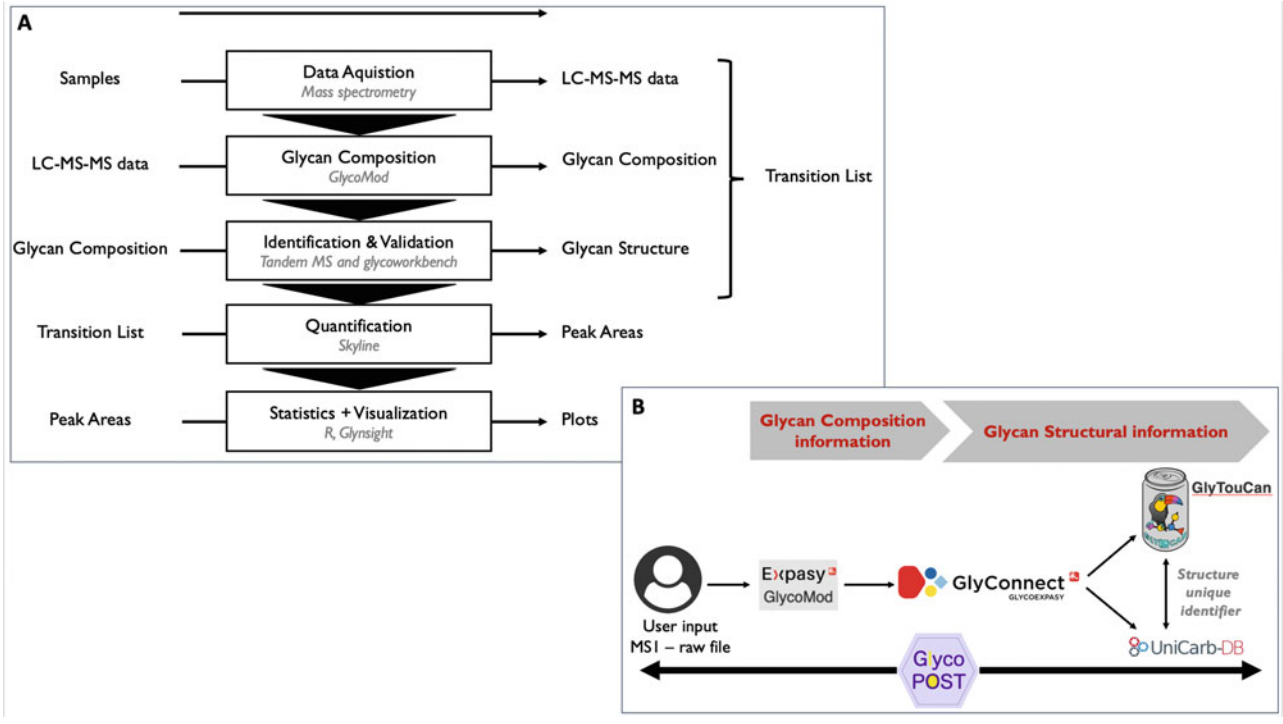

Fig. 6.3 Glycomics workflow. (**a**) The glycomics data analysis was divided into five major procedures. The raw data is acquired on the LC-MS instrument resulting in tandem MS spectra and retention time for each precursor mass selected for fragmentation. The MS1 masses allow for the determination of glycan composition followed by glycan structure identification and validation. The quantification can be performed using Skyline and peak areas can be exported and readily visualized using either R or various other visualization tools such as Glynsight available on the Expasy server. (**b**) MS raw files can be submitted to GlycoPOST including experimental metadata, glycan identification, and quantification results. Glycan composition information can be processed to obtain structural information

software allows for easy deconvolution of the precursor masses. For other instruments, a vendor-specific software package can be used for this purpose. As described below, KNIME (KNIME GmbH) with OpenMS 2.3 can be used for MS1 peak picking and deconvolution.

2. Open the web-based GlycoMod tool with the following parameters:
   (a) All mass values: Monoisotopic
   (b) Mass tolerance: ±0.2 Da (can be adjusted depending upon the mass accuracy of the instrument)
   (c) Ion mode and adduct: Neutral [M]
   (d) *N*-linked oligosaccharide: Reduced oligosaccharides
   (e) Monosaccharide residues present (if known): Underivatized and input the range if known
3. Enter the list of experimental masses.
4. At the bottom, click start GlycoMod.
5. A list of possible glycan compositions is calculated for each mass imputed with a link to GlyConnect.
6. Click on GlyConnect to view possible isomers where each structure is cross-referenced to GlyTouCan and UniCarb-DB.

6.5.1.3 Annotation of MS and MS/MS Glycomics Spectra

1. Launch GlycoWorkbench.
2. Draw the proposed glycan structure using the GlycanBuilder for each precursor mass. Theoretical m/z values can be calculated by configuring the parameters in the "Mass options" available under the "Edit" tab.
3. In silico fragmentation can be computed for selected structures using "compute fragments for selected structure" available in the "Tools" tab.
4. In the "Fragmentation options" pop-up window offers options to select specifications for fragmentation.
5. The results are displayed in a tabular form in the side panel containing all predicted fragments. Different fragments with the same exact mass are represented separately within the details tab. Whereas, the Summary tab contains a condensed form of the information presented within the detail tab.
6. Glycan structures can then be assigned based on glycan fragmentation pathways, in silico fragmentation, observed fragmentation, and precursor mass.
7. Additionally, experimental MS/MS peak lists can also be associated with the computed peak list.
8. Copy the list of fragments m/z and intensities directly from the MS software or export the list first to a spreadsheet using, for example, Microsoft Excel.
9. Next, go to Tools → Annotation → Annotate peaks with fragments from selected structures. A Fragment option window will appear. After setting the parameter, click OK. Then the associated peak list will appear in the right panel.

6.5.1.4 Convert Raw Mass Spectrometry Data to mzXML

1. Open MSconvertGUI.
2. Select the list of files that needs conversion and select the Output directory for the converted files.
3. In the options box below the output directory, adjust the settings to output format = "mzXML," Binary encoding precision = "64-bit," and check the boxes next to "write index," "use zlib compression," and "TPP compatibility."
4. In the filter box, select the subset option and do not change the settings that pop up (default setting).
5. At the bottom right corner, click "start," and wait for your files to finish converting to mzXML.

6.5.1.5 Skyline for Glycomics Quantitation

1. Open Skyline and select either the molecule interface or the mixed interface.
2. Go to the "Setting" tab and select "Transition settings."
3. In the pop-up window, select "Instrument" tab and input Min and Max m/z.
4. Next, in the "Full-Scan" tab, input/select the following parameters depending on the type of instrument used: (a) Isotope peak included (count), (b) Precursor mass analyzer (QIT), (c) Peaks (3), and (d) Resolution (0.3 m/z). The values in parentheses indicate the value used for the analysis.
5. At the bottom, select "Include all matching scans" and click OK.

6.5.1.6 Setting Up Transition List

1. Go to the "Edit" tab, and select Insert → "Transition list."
2. In the pop-up window, select "Molecules" at the bottom and click on the "Columns" button.
3. Select the required columns to set up the transition list. Be sure to include at least (a) Molecule List Name, (b) Precursor Name, (c) Precursor m/z, (d) precursor charge, (e) Explicit retention time, and (f) Explicit retention time window.
4. For large lists of glycans, it is advisable to create the "Transition list" in Excel.
5. The "Transition list" can be easily copied/imported to Skyline using File → Import → "Transition list." Once successful, you will see the Transitions in the Target menu.
6. Now save the File before importing the results (converted mzXML files).
7. Wait for the import to complete. Skyline integrates the area under the curve based on the information provided in the "Transition List." Check if the correct peaks are integrated for each glycan. If required, manual integration can be performed by clicking and dragging the integration window beneath the *x*-axis.

8. Skyline offers various parameters for the quality control of the integrated signals, including mass accuracy, isotopic pattern matching, and retention time which can be conveniently plotted for each sample.
9. Results can be exported using Files → Export → Report function as CSV file.

6.5.2 *Glycopeptide Data Processing for Enriched Immunoglobulins*

The analysis of Ig glycosylation at the glycopeptide level has the advantage that protein-, site-, Ig isotype- and often even subclass-specificity are maintained (Momčilović et al. 2020). General workflows include the affinity purification of the Igs of interest, their tryptic cleavage, and the analysis of the resulting (glyco)-peptide mixture by reverse-phase (RP) liquid chromatography (LC) coupled to high-resolution mass spectrometry (MS). The RP-LC separation of (Ig) glycopeptides results in the retention time clustering of analytes with the same peptide portion but different glycan moieties. Often, distinct retention time clusters are also obtained for glycopeptides with varying degrees of sialylation. Based on this very characteristic and predictable elution behavior of glycopeptides, software packages were developed for the identification [GlycopeptideGraphMS (Choo et al. 2019)] and targeted quantification and quality control [LaCyTools (Jansen et al. 2016)] of glycopeptides derived from purified proteins. Combining these tools with MS2-aided glycopeptide validation resulted in a workflow for the semiautomated identification and quantification of glycopeptides that is highly suitable for high-throughput Ig glycosylation analysis (Lippold et al. 2020). The protocol outlined below is adjusted for data [publicly available via (Bern et al. 2012)] obtained by the RP LC-Orbitrap-MS/MS analysis of tryptic IgG and IgA glycopeptides, Fig. 6.4 (Glycoproteomics workflow).

6.5.2.1 Software Required

1. *Manual inspection of the raw data*: instrumentation proprietary software. In this case, Xcalibur (v. 2.2, Thermo Fisher Scientific).
2. *MS2-based glycopeptide identification*: software package for automated MS/MS assignment of glycopeptides [example can be found in (Abrahams et al. 2020)]. In this case, PMI-Byonic (v. 3.7.13, Protein Metrics), (Bern et al. 2012) Fig. 6.4a.
3. *Data format conversion*: MSConvertGUI (ProteoWizard 3.0 suite, http://proteowizard.sourceforge.net/) (Kessner et al. 2008), Fig. 6.4b.
4. *MS1 peak picking and deconvolution*: KNIME (KNIME GmbH) with OpenMS 2.3 (https://www.openms.de) (Pfeuffer et al. 2017). Details on the installation of these, along with a dedicated workflow to prepare GlycopeptideGraphMS inputs are available as part of the GlycopeptideGraphMS download. (workflow: KNIME_OPENMS_GraphMS_Preprocessing_120318, Fig. 6.4c; https://bitbucket.org/glycoaddict/glycopeptidegraphms/src/master/).

5. *MS1-based glycopeptide identification*: GlycopeptideGraphMS, Fig. 6.4c (v. 2.06, https://bitbucket.org/glycoaddict/glycopeptidegraphms/src/master/).
6. *Glycopeptide quantification and quality control* (QC): LaCyTools, Fig. 6.4d (v 2.0.1, https://git.lumc.nl/cpm/lacytools).
7. Python 3 with the Anaconda package (https://www.anaconda.com/).

6.5.2.2 Glycosylation Site Identification Based on MS2

Byonic can be used to identify the peptides that are present in samples as well as validating the presence of glycopeptides (Fig. 6.4a). There are three inputs: raw files, a protein database, and a glycan database (in this case, Byonic proprietary *N*-linked database).

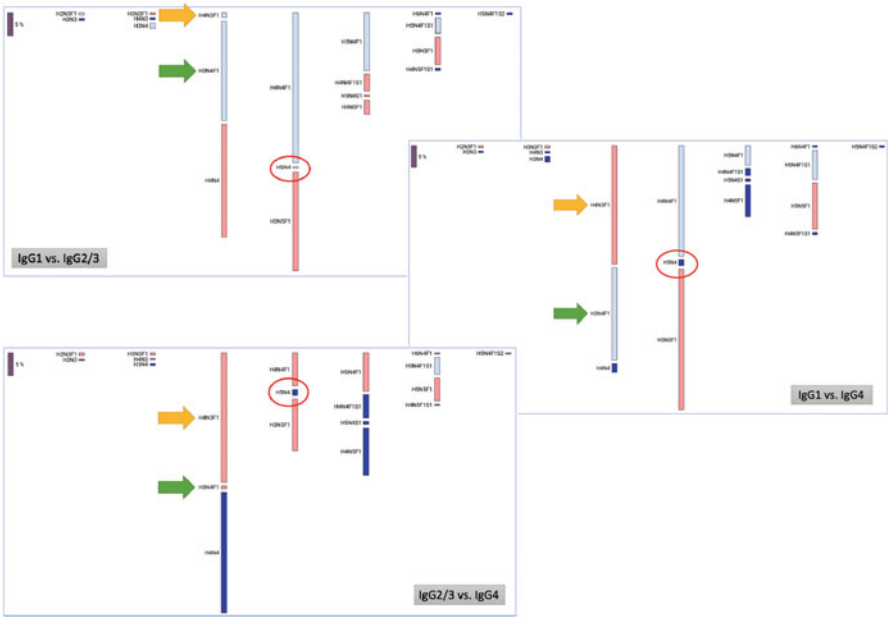

Fig. 6.4 Glycoproteomics workflow. The glycoproteomics dataset was subjected to (**a**) glycosylation site identification by MS2 and (**b**) MS1 feature selection and deconvolution. (**c**) The outcomes of these processes were combined using GlycopeptideGrapMS for extensive glycoform identification and visualization. (**d**) The identified glycopeptides were subjected to a QC step and quantified using LaCyTools

1. Run PMI-Byonic on the raw LC-MS/MS data files with the Homo sapiens UniProt database including 71,591 protein sequences (20,205 from Swiss-Prot and 51,386 from TrEMBL at time of writing). Byonic has an inbuilt glycan database for putative glycan assignments. In this example the "N-glycan 309 mammalian no sodium" glycan list is used. (see Note 1). Individual options are set to:

   (a) C-terminal cleavage of lysine and arginine: true
   (b) Maximum number of missed cleavages: 2
   (c) Precursor mass tolerance: 10 ppm
   (d) Fragment ion mass tolerance: 20 ppm
   (e) Fixed modification: cysteine carbamidomethylation
   (f) Variable modification: methionine oxidation

2. Output from this step is a list of polypeptide cleavage products and glycopeptide identifications. Identify those products of the protein(s) of interest. Include glycopeptide identifications with a Byonic score above 200 (see Note 2) (Fig. 6.4a).

6.5.2.3 Glycoform Identification Based on MS1

GlycopeptideGraphMS can be further used in the identification process to cluster the identified glycopeptides by retention time. These nodes can reveal information on the underlying proteins and glycosylation sites. Advanced (and/or customized) options can be investigated using GlycopeptideGraphMS manual (https://bitbucket.org/glycoaddict/glycopeptidegraphms/).

1. A preprocessing step is required to create input for GlycopeptideGraphMS. Convert the raw data to the mzML format using MSConvertGUI with the following settings:

   (a) Binary encoding precision: 64-bit
   (b) Filters: subset – MS level: 1-1
   (c) Check: write index, use zlib compression, TPP compatibility

2. Load the obtained mzML file in the KNIME OpenMS workflow and define an output directory. Adjust the default settings in the feature finder to account for the m/z range (400–3500) and expected charge states (2–7). Run the file filter, peak picking, feature finder, and de-charging node (Fig. 6.4b). Adjust these settings according to instrumentation.
3. Load the obtained .csv file from the KNIME OpenMS workflow into GlycopeptideGraphMS and format it into the consensus format needed for further processing using the "Format consensusXML" function.
4. Perform the GlycopeptideGraphMS analysis with the following settings (only values deviating from the default are mentioned):

(a) Mass deviation tolerance: 0.02 Da
(b) Maximum subgroup degree: 1 (the lower the value, the higher the noise)
(c) Composition search blocks (see Note 3):

(i) Hexose (Hex, 162.0528 Da, max. 30 s retention time (RT) difference)
(ii) *N*-Acetylhexosamine (HexNAc, 203.0794 Da, max. 30 s RT difference)
(iii) Hexose + *N*-acetylhexosamine (HexHexNAc, 365.1322 Da, max. 30 s RT difference)
(iv) Deoxyhexose (Fuc, 146.0579 Da, max. 20 s RT difference)
(v) *N*-Acetylneuraminic acid (NeuAc, 291.0954 Da, max. 120 s RT difference)

5. The obtained output.csv provides a list of all nodes identified in so-called "subgraphs" (glycopeptides that share the same peptide portion and differ in their glycan content). Entries (rows) with the same value under "refnode" belong to the same subgraph. Use the m/z (mz_node) and retention time (rt_node) of each node to match them to glycopeptides identified during the MS2-based identification (Sect. 5.2.2).
6. Open the "reference node XLSX" which lists one node per identified subgraph. Populate each entry by one MS2-confirmed node per subgraph (see Note 4 and Note 5), indicating the node number (node), m/z (mz_node), retention time (rt_node), the number of the subgraph node with the lowest mass with its m/z (refnode; mz_refnode), the number of the respective monosaccharides present in the identified glycopeptide and the peptide sequence of the identified glycopeptide, as illustrated in Table 6.2.
7. Use the graphical representation option in GlycopeptideGraphMS to visualize the identified subgraphs. Closely related clusters help to identify (unexpected) peptide modifications and glycoforms, Fig. 6.4c which can be used to re-run the glycopeptide (Byonic) search and optimize the output. It also allows the identification of unexpected peptide modifications, such as partial cysteine oxidation (identified on the IgA data in this example).
8. Run the composition predictor in GlycopeptideGraphMS (input: reference node XLSX and output.csv) to obtain a full list of glycoforms, based on the MS1 LC-MS features.
9. Remove the glycoforms from the predicted composition output of GlycopeptideGraphMS that contain negative values in the final glycoform composition for one or more of the composition search blocks (illogical nodes). Use the graphical representation to remove the glycoforms with logical compositions that were only connected to the subgraph via an illogical node.

Table 6.2 Description of an MS2-confirmed node per subgraph, following the GlycopeptideGraphMS analysis

Subgraph	node	mz_node	rt_node	Refnode	mz_refnode	N	H	F	S	Pep
6	989	2658.155	1769.709	989	2658.155	3	4	1	1	SLHVPGLNK

6.5.2.4 Targeted Glycopeptide Quantification

1. Convert the raw data (see Note 6) to the mzXML format using MSConvertGUI with the following settings:
   (a) Binary encoding precision: 64-bit
   (b) Filters: subset – MS level: 1-1
   (c) Check: write index, use zlib compression, TPP compatibility
2. Place the LaCyTools master folder in the working directory.
3. Based on the peptide fragments identified in the previous steps, create text files for the peptide "building blocks" representing the peptide potions, following the format below (example for the IgA2 glycopeptide covering site Asn337). Save the text file as [peptidename].block file and place it in the blocks folder in the LacyTools master folder (Fig. 6.4d).
   TPL.block format:
   (a) mass
   957.5495
   (b) available_for_charge_carrier 0
   (c) carbons 42
   (d) hydrogens 75
   (e) nitrogens 11
   (f) oxygens 14
   (g) sulfurs 0
4. When there is more than one data file to quantify, pick one as the reference file. Create a tab-delimited text file (LaCyTools alignment file) listing the m/z of a minimum of five highly confident (see Note 7) glycopeptide compositions covering the entire retention time range in the first column and the retention time of these features in the reference file in the second column.
5. Create a tab-delimited text file (LaCyTools analyte list) containing the following:
   (a) Column 1: all identified glycopeptides, using the annotation TPL1H5N4F1S1 for the peptide covering IgA2 site Asn337 (TPL), carrying a glycan with five hexoses (H), four *N*-acetylhexosamines (N), one fucose (F), and one sialic acid (S)
   (b) Column 2: the retention time of the center of the LaCyTools glycopeptide cluster the analyte belongs to
   (c) Column 3: the integration m/z window
   (d) Column 4: the glycopeptide cluster retention time window
   (e) Column 5: the minimum charge state for the analyte
   (f) Column 6: the maximum charge state for the analyte
   A dedicated python script is available from [4] to create the LaCyTools analyte list based on the GlycopeptideGraphMS output.

6. In LaCyTools, open the Settings tab and adjust as follows:
   (a) Alignment time window and Alignment mass window: match the variation in retention time over the different runs and the mass accuracy of the analysis, respectively
   (b) Sum spectrum resolution: the average number of data points per 1 m/z in the MS1 spectra
   (c) Charge carrier: proton
   (d) Extraction window padding: 0
   (e) Minimum isotopic fraction: 0.75
   (f) Background detection window: 10 (see Note 8).
7. Open the Batch process tab, select the created LaCyTools alignment file under Alignment file and the LaCyTools analyte list under Reference file. Choose the directory where the .mzXML files are stored and select the following output format: Analyte Intensity, per charge state, background subtracted; Alignment QC; Analyte QC. Run the batch process.
8. The obtained Summary file contains the absolute intensities and QC parameters (mass accuracy, isotopic pattern QC; IPQ, and signal-to-noise; S/N) of all targeted glycopeptides in the selected charge states. In Excel: remove charge states of analytes with an S/N < 9, an IPQ > 0.25 and an absolute ppm error > 10. Sum the areas of the remaining charge states per analyte and correct the total for the exact fraction of the isotope pattern integrated for the respective analyte.
9. To obtain the final data, perform total area normalization per glycopeptide cluster (subgraph). For each data file, sum the absolute intensities of all glycoforms per peptide portion and divide each individual glycopeptide of this cluster by the sum. The obtained values can be used for data visualization and statistical analysis (Fig. 6.4d).

Using this protocol glycopeptide data was quantified for IgG1, IgG2/3, IgG4, IgA2 Asn337 and the joining chain Asn49 (Lippold et al. 2020).

6.5.3 Visualizing Profiles

We rely here on a so-called condensed notation: H for hexose, N for hexosamine, F for fucose, and S for sialic acid. Additionally, small letters designate modifications such as “a” for acetylation, “p” for phosphorylation, and “s” for sulfation, for the most frequent.

6.5.3.1 Structural Dependencies Brought Out by GlyConnect Compozitor

The output of most search engines identifying glycans or intact glycopeptides is usually provided as a list or in a table where the items or the rows are independent. However, the glycan structures or the compositions associated with peptides share

common substructures. The GlyConnect Compozitor tool was developed to visualize these dependencies (Robin et al. 2020). Moreover, this web interface provides the option of comparing experimental results with the content of the GlyConnect database.

The glycoproteomics results of Sect. 5.2 for Ig gamma 4 were selected. Nine compositions were identified on peptide EEQFNSTYR (Asn177 in the corresponding UniProt P01861 entry):

H2N3F1
H3N3F1
H4N3F1
H3N4F1
H4N4F1
H3N5F1
H5N4F1
H5N5F1
H5N4F1S1

To bring out dependencies, Compozitor maps compositions related by shared monosaccharide counts into a graph that eases glycome visualization and comparison. When available, each composition is associated with defined structures. Here is how to generate graphs:

1. Go to glyconnect.expasy.org.
2. On the homepage, click on the orange PROTEIN button to open the Protein View of the database.
3. In the Protein View page, type "P01861" in the search window.
4. In the output of two entries, select/click on entry 770 (Id column), i.e., human immunoglobulin heavy constant gamma 4, for display.
5. The protein page shows all glycan structures reported to be *N*-linked to Asn-177 in ten different references. On the right side of the page, click on the Compozitor link.
6. The Compozitor search fields are pre-filled with the details of the 770 entry. In this case, the protein name is "immunoglobulin heavy constant gamma 4" and the Species is "Homo sapiens," shown in the "Protein" tab.
7. Click on the "Add to selection" button and observe that 33 compositions are recorded in the GlyConnect database, ready for display as "Selection A." Then, move to the "Custom" tab by clicking on it.
8. Copy-paste the nine compositions listed above in the input window. The glycan type is *N*-linked by default. Type "experimental" in the "selection label" window in order to name your input set of compositions.
9. Click again on the "Add to selection" button and observe that the nine compositions are now saved as "Selection B."
10. Display the graph of connected compositions by clicking on the "Compute graph" button.

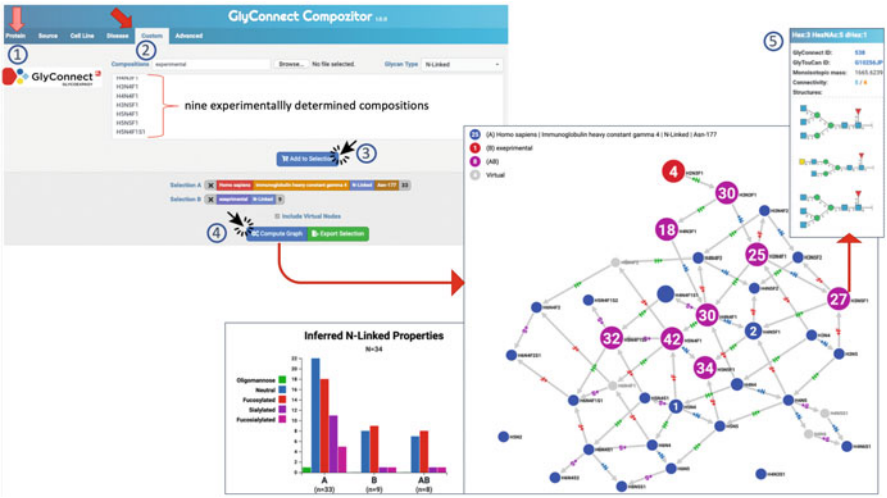

Fig. 6.5 Compozitor output of IgG4 data. The GlyConnect Compozitor tool is accessed directly from the database when querying it to visualize the *N*-glycome of human IgG4. This step is numbered 1 and the data is visualized in the Protein tab. It is labeled "Selection A." Step #2 consists of selecting the Custom tab to input the nine experimentally identified compositions in the glycoproteomics screening of IgG4. During step #3 the input data is recorded as "Selection B" and in step #4, the Compozitor graph is output. Magenta nodes correspond to the match between experimental data and the database content. A window with links to potential structures pops up by mousing over a node in step #5

The graph appears below and shows a well-connected set of 33 + 9 composition nodes that are distinguished by color: blue for the 33 compositions recorded in the database (Selection A), red for the nine input experimental set (Selection B), and magenta for the overlap between the two, as shown in Fig. 6.5. A number contained in a node represents how many glycan structure entries match this composition in GlyConnect. For example, H3N5F1 matched 27 partially or fully defined structures. This information is accessible by mousing over the node to prompt a caption showing the potential structures as shown in Fig. 6.5. The user can then explore the possibilities by clicking on the structure thereby opening the corresponding GlyConnect glycan structure page. The graph shows that the experimental compositions match exactly the mostly documented structures in the database (nodes with highest numbers).

Compozitor allows for "virtual nodes" (in gray) when only one intermediary step is needed to connect isolated nodes. If not for these, the graph would be more scattered.

6.5.3.2 Comparing Profiles with Glynsight

Again, the results of glycopeptide quantification generated by the workflow of Sect. 5.2 were recorded and input in the Glynsight tool (Alocci et al. 2018) for comparing

the expression profiles of each of the IgGs. An example of file in the correct .csv (comma separated values) input format is shown below for IgG4:

```
composition,quantification,
H2N3F1,0.55,
H3N3F1,1.74,
H3N4F1,0.59,
H3N5F1,37.19,
H4N3F1,28.24,
H4N4F1,6.99,
H5N4F1,12.01,
H5N4F1S1,1.04,
H5N5F1,11.64,
```

1. Go to https://glycoproteome.expasy.org/glynsight;
2. In the left menu, click on "Manage experiments" and click on "Import experiments" in the list of tasks that is displayed. Use the "upload files" button to import the .csv files such as the one shown above. The easiest is to give a meaningful name to the file, such as IgG4.csv for the example above.
3. The name of each of the uploaded files is shown in the left menu under "Experiment list." Click on each name of experiment to see the corresponding profile displayed as a bar chart, each bar representing a composition. These are incrementally ordered and structured in columns. All compositions featuring in a column have a constant overall number of monosaccharides. The first column of the profile here contains H3N3 and H2N3F1 (six monosaccharides in total), the next column contains compositions with an overall count of seven monosaccharides, etc. Changing from one experiment to the next immediately brings out the variations.
4. The default display mode is "individual," showing one experiment at a time. To compare profiles, select "differential display" in the Display mode section of the left menu. In this mode, the list of experiments in the left side menu is duplicated to provide the user with the option of selecting an experiment in each of the two lists.
5. Compare IgG1 with IgG2/3, then with IgG4, etc.

Figure 6.6 shows the profile comparisons. Glynsight shows red and blue bars whose height for each composition represents the result of the subtraction. For any given composition, if its intensity in the first experiment selected is higher than in the second, then the bar is blue. In the opposite situation, the bar is red. Brighter blue (resp. red) highlights differences arising from compositions unique to the first (resp. second) experiment while toned down blue (resp. red) shows differences arising from compositions expressed in both experiments but higher in the first (resp. second) than in the second. Grey corresponds to glycans present in other experiments but not in those currently displayed.

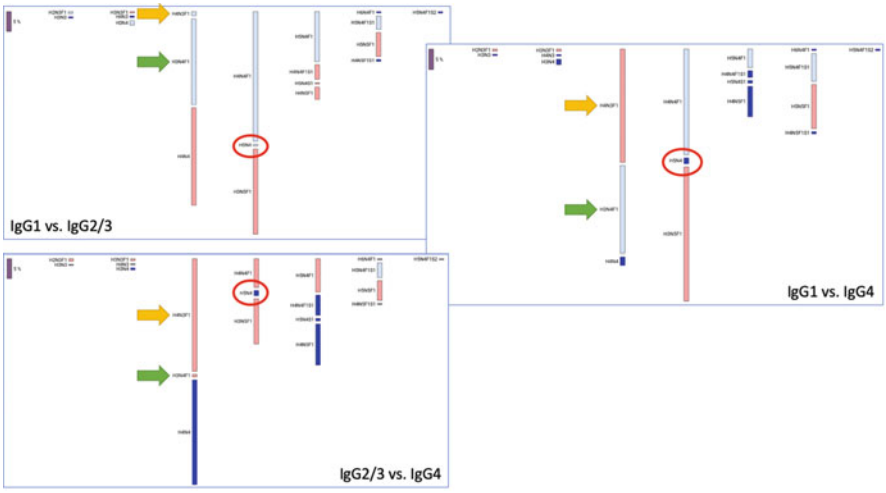

Fig. 6.6 Glynsight comparison of profiles. The comparative profiles of IgG1, IgG2/3, and IgG4 with respect to one another as displayed by Glynsight. Blue (resp. red) bars indicate that expression is higher (resp. lower) in the first profile than in the second. Brighter blue (resp. red) shows differences arising from compositions unique to the first (resp. second) profile while toned down blue (resp. red) shows differences arising from compositions expressed in both profiles but higher in the first (resp. second) than in the second. Green arrows single out the expression of H3N4F1 underexpressed in IgG1, but roughly the same in IgG2/3 and IgG4. Yellow arrows single out the expression of H4N3F1 overexpressed in IgG4 but roughly the same in IgG1 and IgG2/3. Red circles show the constant expression of H5N4 across all sets

The large bars reflect significant differences between the two profiles. The screen capture of the figure is not as visual as when using the software live, since the dynamics of change are not transcribed. Nonetheless, variations are observable. For example, H3N4F1 is overexpressed in IgG1 with respect to both IgG2/3 and IgG4, while it is the same between IgG2/3 and IgG4 (see green arrows in Fig. 6.6). In contrast, H4N3F1 is over overexpressed in IgG4 with respect to both IgG1 and IgG2/3, while it is the same IgG1 and IgG2/3 (see yellow arrows). Another example of constant expression across all sets is that of H5N4 (red circles).

6.6 Conclusion

This chapter has demonstrated the extent of available bioinformatics resources for studying the glycosylation of immunoglobins. On the one hand, both generic and specific databases are interconnected to reflect the growing body of literature on Ig glycosylation and its functional influence. The therapeutic importance of these proteins warrants a special section soon to be developed in the GlyConnect database. On the other hand, the cited software tools have enabled high-throughput and robust analysis, especially for IgG and IgA. In fact, Ig(G)s have quite a head start, compared

to other glycoproteins and many publications already reveal IgG *N*-glycome variations as a reflection of disease states (see recent (Clerc et al. 2018; Martin et al. 2020) for examples). Although the same basic principles apply for the analysis of other glycoproteins, more developments and streamlining of resources are warranted, especially when shifting from isolated proteins to complex samples.

Notes

Glycopeptide data analysis

1. This *N*-glycan library is a good start for Ig samples but should be used with caution as it is far from complete regarding reported *N*-glycan structures. The glycan library used in Byonic can be fine-tuned in an iterative manner after Step 7 in "Glycoform identification based on MS1." In addition, GlyConnect (https://glyconnect.expasy.org/) can be used to construct a protein-specific library based on literature knowledge.
2. According to the Byonic user manual (https://www.proteinmetrics.com/support-information/), the Byonic score ranges from 0 to 1000. A score of 300 is considered good, 400 is very good and scores over 500 are almost sure to be correct. In our experience, lower scores may reflect confident glycopeptide identification, and as further QC will be performed both during the MS1-based identification and the quantification step, using a relatively low threshold score of 200 is justified here.
3. The max. RT differences are highly dependent on the chromatographic setup used and should be optimized based on the specific data analyzed. In general, when using C18 chromatography, the RT difference between two neutral monosaccharides is smaller than between a neutral and a charged monosaccharide.
4. In the likely scenario that more than one node per cluster could be assigned to a specific glycopeptide by MS2, choose the one with the highest confidence. Confidence can be based on Byonic score, manual evaluation of the MS2 data, and prior (literature) knowledge on the expected products.
5. In the scenario that no MS2 confirmed glycopeptide was obtained for any of the nodes in one subgraph, leave the row empty. This can indicate multiple things, including (1) no MS2 spectra were obtained for this glycopeptide cluster, in this case, a targeted MS2 run can be considered; (2) an unknown glycopeptide modification occurred, when this modification is identified Byonic can be run again including it in the search; (3) these nodes were erroneously identified as glycopeptide cluster, exclude the subgraph.
6. To obtain superior quantification precision, data integration should be performed on MS1-only data featuring a high number of data points per chromatographic feature. [4] However, data files including MS2 scans are also suitable, provided that there is a minimum of five MS1 data points per chromatographic feature.
7. While subgraphs or glycopeptide clusters are defined as a group of glycopeptides that share the same peptide portion and differ in their glycan content, in the current context separate LaCyTools glycopeptide clusters were defined for

glycopeptides with the same peptide portion but different sialic acid contents. In general, the more clusters defined, the longer LaCyTools processing time, but the lower the chance of analyte overlaps that interfere with the quantification.
8. Please find an in-depth explanation of all settings in [3] and [5]. The definition of the isotopic pattern QC (IPQ) changed between the initially published version of LaCyTools and the current and is now defined as the sum of the relative intensity deviation per isotope.

Visualization tools

9. Several of the images showing a Compozitor graph in figures have been slightly modified from the raw output to disentangle the network which is generated by the D3.js (https://d3js.org) library. This transformation is made easy since any node can be dragged wherever a user wishes in the space of the browser window by maintaining the mouse over it. Consequently, paths can be shortened or stretched upon user actions.
10. Compositions can be exported in a text file as soon as they have been added to the selection and before computing the graph ("Export selection" button). They can be selectively exported once the graph is computed ("Export" button). The graph can be exported in the .svg format.
11. The "Zoom in" and "Zoom out" buttons have obvious purposes and the former operation is supplemented by the "Zoom on" window located in the top right corner; typing a specific composition in this window will trigger zooming in and centering the graph on the corresponding node.
12. Mousing over a bar of the bar plot of glycan properties highlights all nodes that are counted in that bar to show where they are located in the graph.

Compliance with Ethical Standards

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Ethical Approval This chapter does not contain any studies with human participants performed by any of the authors.

Conflict of Interest Frédérique Lisacek declares that she has no conflict of interest.
Kathirvel Alagesan declares that he has no conflict of interest.
Catherine Hayes declares that she has no conflict of interest.
Steffen Lippold declares that he has no conflict of interest.
Noortje de Haan declares that she has no conflict of interest.

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Part II
Biosynthesis and Regulation

Chapter 7
N-Glycan Biosynthesis: Basic Principles and Factors Affecting Its Outcome

Teemu Viinikangas, Elham Khosrowabadi, and Sakari Kellokumpu

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Abstract Carbohydrate chains are the most abundant and diverse of nature's biopolymers and represent one of the four fundamental macromolecular building blocks of life together with proteins, nucleic acids, and lipids. Indicative of their essential roles in cells and in multicellular organisms, genes encoding proteins associated with glycosylation account for approximately 2% of the human genome. It has been estimated that 50–80% of all human proteins carry carbohydrate chains—glycans—as part of their structure. Despite cells utilize only nine different monosaccharides for making their glycans, their order and conformational variation in glycan chains together with chain branching differences and frequent post-synthetic modifications can give rise to an enormous repertoire of different glycan structures of which few thousand is estimated to carry important structural or functional information for a cell. Thus, glycans are immensely versatile encoders of multicellular life. Yet, glycans do not represent a random collection of unpredictable structures but rather,

T. Viinikangas · E. Khosrowabadi · S. Kellokumpu (✉)
Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland
e-mail: sakari.kellokumpu@oulu.fi

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a collection of predetermined but still dynamic entities that are present at defined quantities in each glycosylation site of a given protein in a cell, tissue, or organism.

In this chapter, we will give an overview of what is currently known about *N*-glycan synthesis in higher eukaryotes, focusing not only on the processes themselves but also on factors that will affect or can affect the final outcome—the dynamicity and heterogeneity of the *N*-glycome. We hope that this review will help understand the molecular details underneath this diversity, and in addition, be helpful for those who plan to produce optimally glycosylated antibody-based therapeutics.

Keywords Endoplasmic reticulum · Golgi apparatus · *N*-glycosylation · Organelle homeostasis

List of Abbreviations

Alg	Yeast Asparagine-linked glycosyltransferase
ALS	Amyotrophic lateral sclerosis
Asn	Asparagine
BiP	Binding protein
CDG	Congenital disorders of glycosylation
CEA	Carcinoembryonic antigen
CMAH	Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase
CMP-Sia	Cytidine-5′-monophospho-*N*-acetylneuraminic acid
COPII	Coat protein complex II
Dol	Dolichol
Dol-P	Dolichol phosphate
Dol-P-Glc	Dolichol monophosphate glucose
Dol-P-Man	Dolichol monophosphate mannose
Dpagt1	Dolichyl-phosphate *N*-acetylglucosamine-phosphotransferase 1
EDEM	ER degradation enhancing mannosidase-like protein
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ERGIC-53	ER-Golgi intermediate compartment 53 kDa protein
ERManI	ER mannosidase I
ERp57	Endoplasmic reticulum resident disulfide isomerase
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	*N*-acetyl-D-galactosamine
GCNT2	*N*-acetyl-lactosamine-β-1,6-*N*-acetylglucosaminyltransferase
GDP-Man	Guanosine-5′-diphosphate-α-D-mannose
Glc	Glucose
GlcNAc	*N*-acetyl-D-glucosamine

GLUT-2	Glucose transporter type 2
HIF	Hypoxia-inducible factor
LacNAc	*N*-Acetyl-D-lactosamine
LLO	Lipid-linked oligosaccharide
LMAN1	Lectin mannose-binding 1
M6P	Mannose-6-phosphate
Man	Mannose
MCFD2	Multiple coagulation factor deficiency protein 2
MGAT	Mannosyl-glycoprotein-*N*-acetylglucosaminyltransferase
MPR	Mannose-6-phosphate receptor
NAGPA	*N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase
Neu5Ac	5-*N*-Acetylneuraminic acid (sialic acid)
Neu5Gc	5-*N*-Glycolylneuraminic acid (sialic acid)
OS-9	Protein OS-9; amplified in osteosarcoma 9
OST	Oligosaccharyltransferase complex
ROS	Reactive oxygen species
Rtf1	A transmembrane protein encoded by the yeast Rft1 gene
Sec61	A three-subunit (Sec61α, Sec61β, and Sec61γ) protein translocation complex
Ser	Serine
SERCA2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Sia	Sialic acid
SPCA1/2	Secretory pathway Ca(2+)-ATPase type 1and 2
ST3Gal1	Beta-galactoside α-2,3-sialyltransferase 1
ST3Gal3	Beta-galactoside α-2,3-sialyltransferase 3
ST6Gal1	Beta-galactoside alpha-2,6-sialyltransferase 1
STT3A/B	Catalytic A and -B subunits of the oligosaccharyltransferase complex
TGN	*Trans*-Golgi network
Thr	Threonine
TMEM165	Transmembrane protein 165
UDP-GlcNAc	Uridine-5′-diphosphate-*N*-acetyl-α-D-glucosamine
UDP-Gal	Uridine-5′-diphosphate-α-D-galactose
UDP-Glc	Uridine-5′-diphosphate-α-D-glucose
UGGT	UDP-glucose-glycoprotein glucosyltransferase
VIP 36	Vesicular integral-membrane protein 36
XTP3-B	XTP3-transactivated gene B lectin

7.1 Introduction

Asparagine-linked (*N*-linked) glycosylation is an essential protein modification, affecting a number of basic cellular processes such as protein folding, its half-life, trafficking and immunogenicity as well as its interactions between cells, cells and extracellular matrix components or pathogens (Varki and Gagneux 2017). In all eukaryotic cells, *N*-glycans are synthesized in two specialized organelles, the endoplasmic reticulum (ER) and the Golgi apparatus. Together, these organelles harbor dozens of functionally distinct glycosyltransferases and glycosidases that sequentially modify the growing oligosaccharide chain (Kornfeld and Kornfeld 1985; Dunphy 1985; Spiro 2002; Rabouille et al. 1995). Yet, it is much less clear how this sequence of enzymatic reactions is orchestrated to guarantee faithful synthesis of *N*-glycans, considering that enzymes do not use any template, can compete with each other for the same substrate and/or the acceptor, and even localize in the same Golgi compartment. Another puzzling issue is an intrinsic microheterogeneity of glycans made by the cell. For example, an *N*-glycan attached to a specific asparagine of a given protein can be different from an *N*-glycan attached to the same site in another protein molecule. Distinguishing this "background noise" from dynamic changes that are functionally important, e.g., during embryonic development, cell differentiation, and aging can sometimes be problematic. Nevertheless, unlike other polymerization events in the cell, glycosylation apparently need not be a high-fidelity system, since cells normally tolerate such microheterogeneity without facing problems in cell survival or proliferation.

The other side of the coin is that this variation can sometimes lead to a devastating disease. Congenital disorders of glycosylation (CDGs) are a rare, yet diverse group of serious, often multiorgan diseases characterized by defects in glycosylation (Freeze et al. 2014; Francisco et al. 2020). More than 140 different CDG syndromes are known as of today, the severity of which varies from prenatal death to survival into adulthood with a relatively normal life span. Disturbed *N*-glycosylation forms the largest group of the CDGs. It is divided into two groups (Type I and Type II) based primarily on the genetic defect and the site it is affecting. Type I CDGs are characterized by defects in the synthesis of *N*-glycans in the endoplasmic reticulum (ER), while the Type II CDGs have problems in their processing in the Golgi apparatus. In addition to CDGs, glycosylation changes play an important role in many other human diseases including autoimmune diseases, inflammation, tumorigenesis, and its progression (Reily et al. 2019). Yet, the underlying mechanistic details that cause these changes are incompletely understood, as are also the reasons why some changes lead to disease and some do not. Partly, this is due to the dynamic and variable nature of the glycan themselves, their cell- and tissue-specific expression (Medzihradszky et al. 2015) as well as the lack of tools that would allow glycan editing at will in a specified glycosylation site or protein itself.

7.2 Biosynthesis of *N*-Glycans in the Endoplasmic Reticulum

7.2.1 Building Blocks for N-*Glycan Synthesis*

The early steps in *N*-glycan biosynthesis in the endoplasmic reticulum (ER) are conserved in all three domains of life (Dell et al. 2010), whereas their processing and maturation differ markedly. All *N*-glycans share a common core structure (asn-GlcNAc2Man3-) which is further elongated in a species- and tissue-specific manner (Medzihradszky et al. 2015) by adding a few other subterminal or terminal sugar residues to the core structure. Depending on the sugar residue and the linkage type used, these additions can significantly influence the structure of the *N*-glycan (Medzihradszky et al. 2015). The main sugar residues utilized as building blocks are *N*-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal), fucose (Fuc), and sialic acid (*N*-acetylneuraminic acid (Neu5Ac) being the predominant form), of which the latter two act as chain-capping residues. In some instances, *N*-acetylgalactosamine (GalNAc) residues can be used to construct an *N*-glycan. Glucose (Glc) residues are also temporarily incorporated into the growing *N*-glycan during its synthesis in the ER, yet they are invariably removed as glucose residues have not been detected in a mature *N*-glycan isolated from cultured cells or tissues (Zuber et al. 2000). Occasionally, mature *N*-glycans can also be modified by the addition of sulfate or phosphate, generating determinants that modulate cell adhesion or glycoprotein localization in the cells. Interestingly, despite the deletion of the CMAH gene (needed for *N*-glycolylneuraminic acid (Neu5Gc) synthesis) 3 million years ago, this neuraminic acid variant is still regularly detected in trace amounts in human glycans (Angata and Varki 2002). This is due to dietary consumption of Neu5Gc-containing animal products (e.g., red meat and dairy products) and its incorporation into newly synthesized glycans (Banda et al. 2012). Perhaps unsurprisingly, the highest Neu5Gc levels are detected in epithelial and endothelial cells that line the intestine and blood (and lymph) vessels, respectively.

7.2.2 Precursor Synthesis and Its Attachment to Nascent Polypeptide Chains

The *N*-glycosylation of nascent polypeptides in the ER lumen relies on the prior assembly of a lipid-linked oligosaccharide (LLO) precursor (Glc_3Man_9-$GlcNAc_2$) onto a membrane-embedded dolichyl phosphate (Dol-P) carrier (Fig. 7.1). This set of events is orchestrated by the Alg-family of ER-localized, membrane-associated glycosyltransferases (Kelleher et al. 2007). They stepwise assemble the LLO using nucleotide sugars (UDP-GlcNAc, GDP-Man, Dol-P-Man, and Dol-P-Glc) as donor substrates. The LLO assembly begins on the cytoplasmic face of the ER membrane by the formation of a $GlcNAc_2$-PP-Dol intermediate from GlcNAc-1-phosphate and

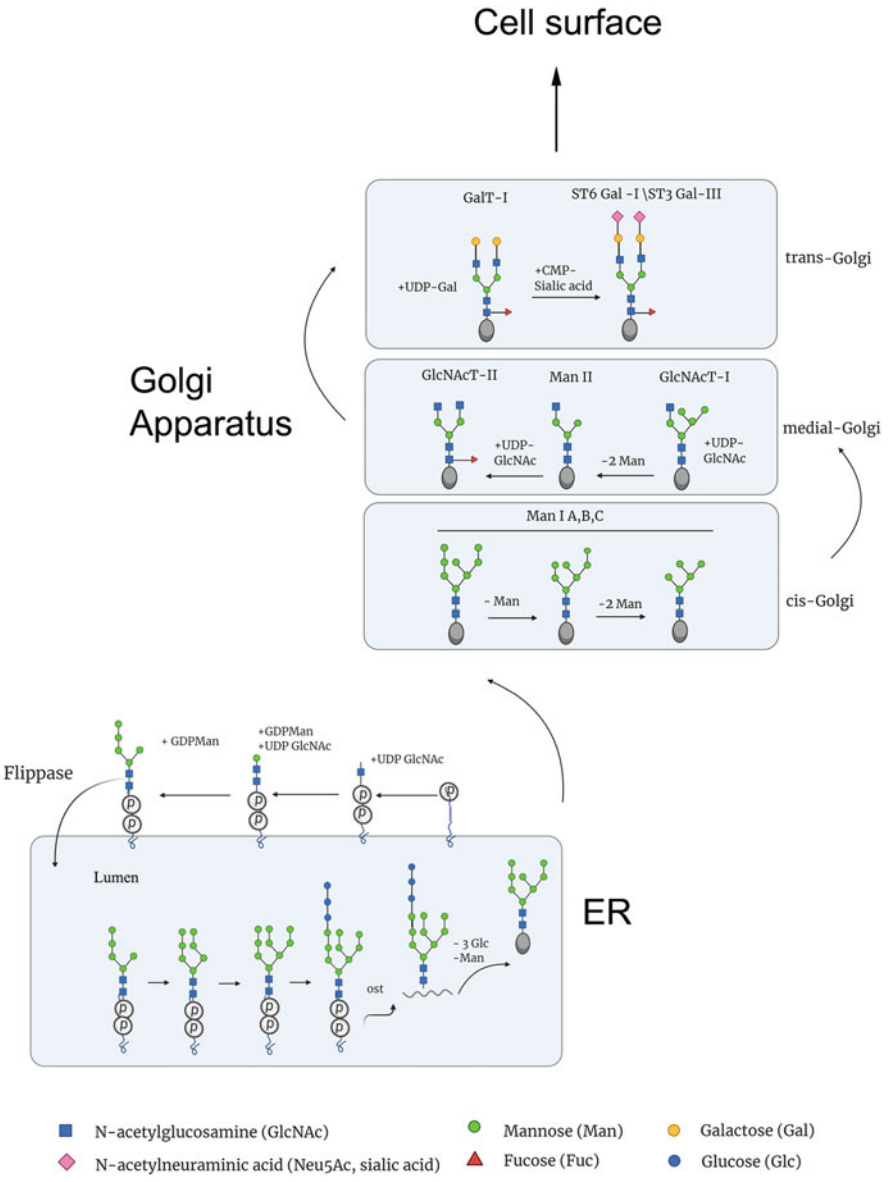

Fig. 7.1 A schematic representation of the *N*-glycan biosynthetic pathway in the ER and the Golgi apparatus. The figure shows the gradual maturation of an *N*-glycan and the various steps involved. For more details, please see the text

GlcNAc. These additions are catalyzed by Dpagt1 (Alg7) and Alg13p/Alg14p UDP-GlcNAc-transferases, respectively. The three enzymes exist as hexamers with a stoichiometry of 2:2:2 (Noffz et al. 2009). Alg14 appears to be the central

unit, capable of recruiting other enzymes to the complex (Lu et al. 2012). Next, ER mannosyltransferases (Alg1, Alg2, and Alg11) that also form complexes with each other (Gao 2004) add five mannose residues from GDP-Man donors to form a $Man_5GlcNAc_2$-PP-Dol intermediate. Thus, LLO precursor synthesis on the cytoplasmic face of the ER membrane involves three main enzyme complexes, one formed by Dpagt1/Alg13/Alg14 and the other two either by Alg1/Alg2 and Alg1/Alg11. This arrangement likely ensures that each mannose residue will be linked correctly to the precursor despite the coexistence of several competing enzymes on the same membrane.

The next step involves translocation of the $Man_5GlcNAc_2$-PP-Dol intermediate into the ER lumen, a process that is thought to be mediated by a protein termed as the Rft1, but it is still uncertain whether it acts as a bonafide flippase protein (Helenius et al. 2002). In the ER lumen, mannosyltransferases (Alg3/Alg9/Alg12) and glucosyltransferases (Alg6/Alg8/Alg10) further elongate the LLO precursor by attaching four additional mannose residues and three glucose residues, respectively. This completes the precursor synthesis and yields the $Glc_3Man_9GlcNAc_2$-PP-Dol structure, which will be used later as the donor substrate for *en bloc* transfer of an *N*-glycan to a suitable polypeptide chain. It is noteworthy that unlike the initial catalytic steps on the cytosolic face of ER, the completion of the LLO precursor synthesis in the ER lumen does not use nucleotide sugars as donors. Rather, membrane-embedded Dol-P-Man and Dol-P-Glc are used as sugar donors in this case. Their synthesis takes place also on the cytoplasmic side of the ER membrane (from GDP-Man and UDP-Glc, respectively) before they are translocated (flipped) to the luminal side (Helenius et al. 2002).

The most preferred acceptor asparagine residues for *N*-glycosylation are the ones within the Asn-X-Ser/Thr motif (where X $\neq$ proline) (Zielinska et al. 2010). Of these two, the Asn-X-Thr sequon is preferred over Asn-X-Ser, mainly because the interaction between the side chain methyl group of threonine and the asparagine-lysine (NK) motif in the binding pocket of the oligosaccharyltransferase (OST) increases the stability of the complex (Kasturi et al. 1995, 1997; Medus et al. 2017). The identity of the amino acid X and flanking amino acids also contribute to the glycosylation of a given sequon. In addition, the position of the sequon within the polypeptide, the secondary and tertiary structure of the protein, and its final destination in a cell can impair or enhance the likelihood of whether that site becomes glycosylated or not (Rao and Wollenweber 2010). Thus, the presence of sequons alone cannot be used as an adequate predictor of *N*-glycosylation. Indeed, roughly one-third of the identified sequons in secreted glycoproteins remain non-glycosylated (Schulz 2012).

The transfer of the completed precursor oligosaccharide is catalyzed by ER membrane-localized OST complex. It is an octamer consisting of a single catalytic subunit and seven accessory subunits, each important for optimal glycosylation efficiency. Most multicellular animals (sponges are an exception) possess two such complexes due to an ancient duplication of the gene encoding the catalytic subunit. The STT3A and STT3B (for OST-A and OST-B complexes, respectively) have different kinetic properties, acceptor substrate preferences, and partially

non-overlapping roles in glycosylation (Shrimal et al. 2013a). The accessory subunit compositions between the two complexes also differ. OST-A complex associates with Sec61 core components of the ER translocon complex and co-translationally glycosylates the nascent polypeptide in accessible sequons during polypeptide chain translocation into the ER lumen. Sequons within the last ~50–55 residues of the C-terminus are, however, inside the translocon and hence inaccessible for STT3A. Instead, STT3B transferase in OST-B complexes can posttranslationally add *N*-glycans to such sequons. It also can use internal sequons that are skipped by the STT3A as acceptors (Lu et al. 2018; Shrimal et al. 2013b). Often, these include closely spaced sequons adjacent to signal cleavage site or sequons with cysteine residues nearby or inside the motif (i.e., the N-C-T/S motif (Shrimal et al. 2013a).

7.2.3 N-*Glycan Processing in the ER and Quality Control*

The newly attached $Glc_3Man_9GlcNAc_2$ *N*-glycan structure is further modified once the polypeptide is translocated to the ER lumen and begins to fold. The first step involves the removal of the terminal glucose residue by a transmembrane enzyme α-glucosidase I. The second glucose is then rapidly removed by the soluble α-glucosidase II (Grinna and Robbins 1979; Janssen et al. 2010) The resulting mono-glucosylated glycan is a preferred ligand for the carbohydrate-recognizing molecular chaperones calnexin and calreticulin. These chaperones readily associate also with the protein ERp57 (Ruddock and Molinari 2006), a disulfide isomerase that catalyzes the formation of inter- and intramolecular disulfide bonds, thereby helping proper folding of the nascent glycoprotein. Calnexin binding appears to happen irrespective of the folding state of the glycoprotein (Zapun et al. 1997), suggesting that it most likely interacts with the nascent polypeptide as soon as it arrives in the ER lumen. During the folding process (Fig. 7.2), the last glucose residue is removed by the α-glucosidase II. Unfolded or misfolded proteins display exposed hydrophobic patches that are recognized by UDP-glucose-glycoprotein glucosyl-transferase (UGGT) (Caramelo et al. 2003), an enzyme that can glucosylate the same mannose residue again in that *N*-glycan. By doing so, it recreates the $Glc_1Man_9GlcNAc_2$ structure that is again acted upon by the chaperone-disulfide isomerase complex. This removal and re-addition of glucose residues can continue for several cycles until the protein is properly folded. Once this is achieved, the glycan is finally trimmed by ER mannosidase I (ERMan1) that removes the terminal mannose residue from the middle branch of the *N*-linked oligosaccharide. The resulting $Man_8GlcNAc_2$ structure then can be recognized by the ERGIC-53 (LMAN1), a mannose-specific lectin of the LMAN1/MCFD2 cargo receptor complex (Zheng et al. 2010), thereby facilitating packaging and transport of the native *N*-glycosylated glycoprotein into COPII (Coat Protein complex II)-coated vesicular carriers that ferry cargo from the ER to the Golgi via the ER-Golgi intermediate compartment (ERGIC) (Hanna et al. 2018; Peotter et al. 2019).

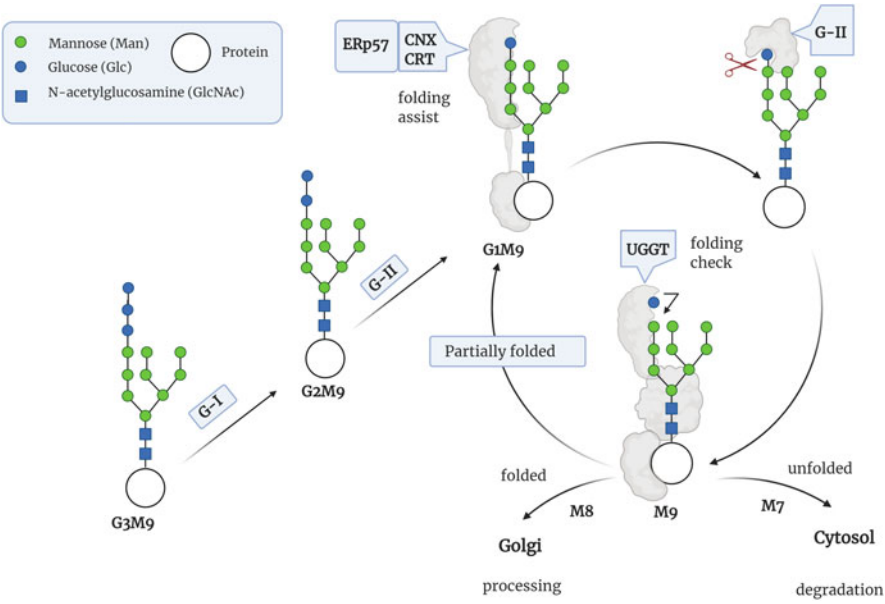

Fig. 7.2 A glycan-based quality control system in the ER that distinguishes correctly folded glycoproteins from unfolded or misfolded ones. *CNX* calnexin, *CRT* calreticulin, *G-I-II* α-glucosidases I and II

An ER stress caused by various factors (e.g., altered calcium homeostasis, redox state and glucose deprivation or mutations) is characterized by accumulation of misfolded or unassembled proteins in the ER and can be detrimental to cell viability. Metazoan cells can, however, cope with this stress by launching an ER stress response that suppresses the rate of translation and increases the expression of molecular chaperones to ease protein folding in the ER. In acase these maneuvers still fail, terminally misfolded glycoproteins will be directed to degradation via an ER-associated degradation (ERAD) pathway (Benyair et al. 2015). It starts when ERMan1 mannosidase and EDEM (ER degradation enhancing mannosidase-like) proteins (EDEM1/2/3 in mammals) are recruited to cleave off two mannose residues (instead of one) from an *N*-glycan. As a result, the glycan becomes unrecognizable by ERGIC-53, thereby preventing glycoprotein transport to later secretory compartments. The exposed α(1,6)-linked mannose in the $Man_7GlcNAc_2$ structure is now recognized by the OS-9/XTP3-B lectin complex that directs the bound glycoprotein to the transient ERAD (ER-associated degradation) protein complex at the ER membrane with ubiquitin ligase activity. ERAD complex then ensures that the glycoprotein is returned back to the cytoplasmic side of the ER membrane by tagging it for proteasomal degradation through ubiquitination (Benyair et al. 2015).

The other route for degrading of misfolded glycoproteins relies on malectin, a membrane-associated, ER stress-induced lectin first identified in 2008 (Schallus et al. 2008). It is highly conserved in metazoans (Yang et al. 2018) and it shows

high specificity toward di-glucosylated *N*-glycans ($Glc_2Man_9GlcNAc_2$) (Schallus et al. 2008, 2010). Malectin forms a stable complex with ribophorin I, a subunit of the OST complex with proposed chaperone activity based on its ability to recognize misfolded protein backbones (Qin et al. 2012; Galli et al. 2011) This complex seems to act as an early intervention mechanism for detecting and capturing nascent non-native *N*-glycoproteins before it delivers them to proteasomal degradation if initial attempts to fold will fail (Stanley 2016). Whether this malectin-ribophorin I-mediated removal mechanism involves a unique retro-translocation machinery different from the ERAD machinery is currently unclear.

7.3 *N*-Glycan Processing in the Golgi Apparatus

Correctly folded glycoproteins entering *cis*-Golgi compartment carry typically an *N*-glycan with eight mannose residues left ($Man_8GlcNAc_2$). While some *N*-glycans may exit the Golgi without being modified, their proportion is normally low in humans (Lee et al. 2014). Partly, this is due to a presence of a quality control mechanism that is present in the Golgi. Golgi membranes harbor a mannose-binding lectin VIP36, that can recycle high mannose type *N*-glycans back to the ER (Lee et al. 2014). In support of this, VIP36 also interacts with the ER-localized BiP chaperone (Nawa et al. 2007). By doing so, VIP36 can halt the secretion of improperly glycosylated glycoproteins to post-Golgi compartments. Another mechanism to prevent high mannose type *N*-glycoproteins from passing through the Golgi takes over when a mono-glycosylated *N*-glycan ($Glc_1Man_9GlcNAc_2$) carrying glycoprotein arrives in the Golgi. The glycan part is cleaved internally by the Golgi endo-α-mannosidase between the two Man residues of the Glcα1–3Manα1–2-Manα1–2 moiety, thereby yielding a $Man_8GlcNAc_2$ isomer that is different from that produced by ERMan1 in the ER (Thompson et al. 2012). Interestingly, experimental evidence also suggests that the calreticulin-based glycoprotein quality control may be functional also in the Golgi compartment, as calreticulin was found to co-localize with endo-α-mannosidase in the ERGIC and *cis*/medial-Golgi compartment at least in cultured rat liver cells (Zuber et al. 2000).

Normally, the vast majority of *N*-glycans are processed in the Golgi to complex and/or hybrid type *N*-glycans by a distinct set of glycosidases and *N*-acetylglucosaminyltransferases, also termed as MGAT1–5 (Kellokumpu et al. 2016; Khoder-Agha et al. 2019a). The processing involves complex mutual interplay between the MGAT homomers and heteromers, mannosidase II (ManII) acting as a central hub (Khoder-Agha et al. 2019a). Thus, upon arriving in the Golgi, ER-derived MGAT homomers form heteromeric complexes not only with other MGATs but also with relevant UDP-*N*-acetylglucosamine transporters. Thereby, they organize into multienzyme/multi-transporter assemblies in the Golgi membranes. Their interplay likely involves either distinct or dynamic complexes (Khoder-Agha et al. 2019a) to facilitate efficient processing and branching of *N*-glycans in the *cis*- and medial-Golgi.

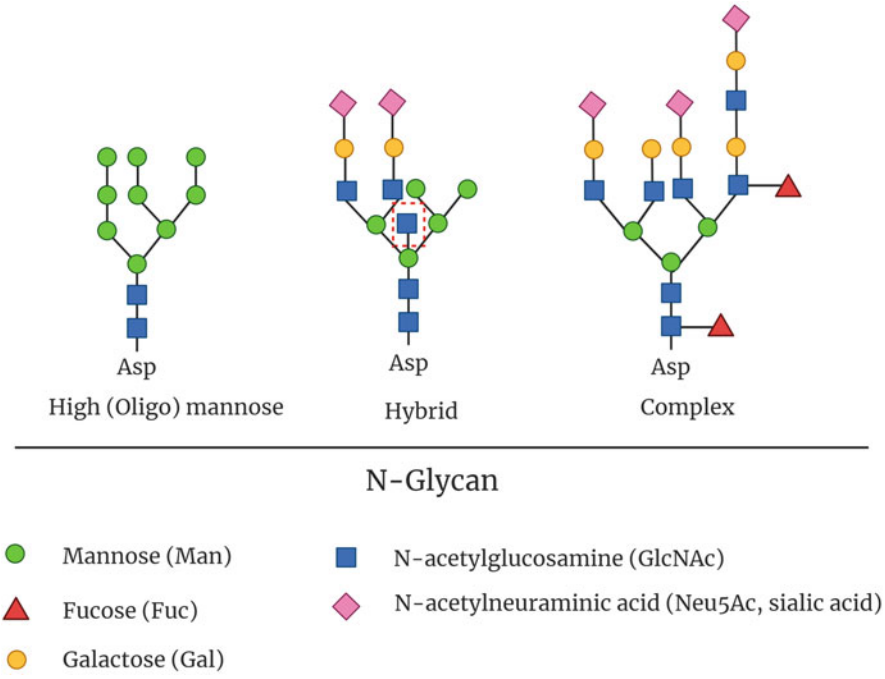

Fig. 7.3 Three examples depicting the main *N*-glycan types present on the cell surface in higher eukaryotes. High-mannose type *N*-glycan is characterized by having not undergone any processing in the Golgi compartment. Hybrid-type *N*-glycan typically has only one branch that has been processed in the Golgi. Bulky complex-type *N*-glycans in turn have two to five branches that are made and terminated in the medial and *trans*-Golgi cisternae

The processing begins in the *cis*-Golgi by the removal of three mannose residues to yield the $Man_5GlcNAc_2$ structure. Golgi mannosidases IA-C are responsible for the cleavages. Then, the first GlcNAc is added by MGAT1, using nucleotide-activated *N*-acetylglucosamine as a donor substrate. Once this GlcNAc is added, two additional mannose residues are removed by the Golgi α-mannosidase II. This creates a scaffold for MGAT2 to add a second GlcNAc to the exposed mannose residue, yielding a precursor for all complex-type *N*-glycans. MGAT4 and MGAT5 can then initiate the synthesis of third and fourth GlcNAc branches, respectively. Alternatively, MGAT3 can add a bisecting GlcNAc at the tri-mannosyl core structure (Fig. 7.3, middle). If this bisecting GlcNAc is added before MGAT4 and MGAT5 have added theirs, the synthesis of the third and fourth GlcNAc branches by MGAT4 and MGAT5 is halted (Kizuka and Taniguchi 2018). Bisecting GlcNAc also cannot be further elongated with any other sugar residue. Its addition also significantly alters the conformation of an *N*-glycan and suppresses the addition of terminal sugar residues such as sialic acid and fucose. The human natural killer-1 epitope (HSO3-3GlcAβ1-3Galβ1-4GlcNAc), a sulfated trisaccharide structure that is extensively expressed in the nervous system, is another terminal epitope suppressed

by the bisecting GlcNAc (Nakano et al. 2019). Bisecting GlcNAc is also known to inhibit α-mannosidase II, suggesting that this addition may be one reason for the synthesis of hybrid type *N*-glycans. Yet, not all hybrid-type *N*-glycans contain a bisecting GlcNAc. It is, therefore, possible that rapid elongation of the first GlcNAc branch by galactose can also inhibit the necessary removal of two terminal mannoses by α-mannosidase II and thus, the build-up of the other GlcNAc branches.

Normally, the GlcNAc branches are further elongated by adding galactose, *N*-acetylglucosamine, and sialic acid. Galactose is added to the GlcNAC nearly always with the β(1,4)-linkage. This structure, termed *N*-acetyllactosamine (LacNAc), can be repeated several times in one branch, forming poly-LacNAc structures. Poly-LacNAc motifs in turn can act as substrates for making additional branches to the antennae. This is done by a set of special enzymes called GCNT2s A-C (Dimitroff 2019) by adding extra GlcNAc residues with the β(1,6)- linkage to internal galactose residues. These GlcNAc residues can also be subsequently elongated by β(1,4)-galactosyltransferases to form additional LacNAc structures. This kind of branched *N*-glycan is termed an I-branched glycan. They are most frequently found in adult erythrocytes, mucosal epithelia, and cells of the eye and olfactory bulb (Dimitroff 2019). GalNAc residues are also occasionally found in *N*-glycans of mammals forming LacdiNAc (GalNAcβ(1,4)GlcNAc) type structures.

The antennae are often capped with sialic acid by various sialyltransferases. This blocks further elongation of the branches except in the case of polysialylation. Polysialylated *N*-glycans are commonly detected in neural cell adhesion molecules (NCAMs) of the nervous system (Kiss and Rougon 1997). Fucose is another residue that cannot be elongated further. It can be added by specific Golgi fucosyltransferases either to the asparagine-linked GlcNAc to produce the "core fucosylated" *N*-glycan, or to GlcNAc residues of the antennae.

In specific cases, sugar residues of the antennae can undergo further modifications such as sulphation, phosphorylation, and *O*-acetylation (Klein and Roussel 1998; Wang et al. 2017). For example, lysosomal acid hydrolases carry *N*-glycans with a phosphate that directs the enzymes to lysosomes. Lysosomal enzymes share common conformational lysine-containing motifs that are recognized by the *cis*-Golgi-localized GlcNAc-1-phosphotransferase enzyme. In the first catalytic step, GlcNAc-1-phosphotransferase transfers GlcNAc-1-P from UDP-GlcNAc to the C6 hydroxyl group of selected mannose residue present in the high mannose-type *N*-glycan (Oh 2015; Qian et al. 2010). In the second step, *N*-acetylglucosamine-1-phosphodiester α-*N*-acetyl-glucosaminidase (NAGPA) cleaves the GlcNAc, leaving only the phosphate group linked to the mannose. Man-6-phosphate (M6P) tag is the ligand for transmembrane Man-6-P receptors (MPRs) residing in the *trans*-Golgi network (TGN). Once recognized by the MPR, the receptor escorts the lysosomal hydrolase with its ligand to endosomes and eventually to lysosomes in clathrin-coated vesicles. In lysosomes, the enzyme is released at low pH and the receptor is recycled back to the *trans*-Golgi. Mutations that impair tagging of mannose with phosphate lead to lysosomal storage diseases, a group of over 70 rare diseases characterized by accumulation of macromolecules in lysosomes (Xu et al. 2016).

7.3.1 N-*Glycosylation of Immunoglobulins*

N-glycosylation is also an important modification of all immunoglobulin isotypes and contributes affecting their binding characteristics and effector functions. Although their synthesis might not be different in any way from other *N*-glycans, there are some special issues that are worth discussing. *N*-glycans attached to immunoglobulin G (IgG) are best characterized owing to IgG abundance in the serum and successful production of many IgG-based therapeutic antibodies by the biopharma industry. IgG *N*-glycans are typically found in the Fc region but a minor proportion (15–25%) of serum IgG can contain *N*-glycans within their variable domains. These so-called "Fab glycans" differ from the Fc region *N*-glycans by having a higher proportion of terminally galactosylated and sialylated *N*-glycans with a bisecting GlcNAc, while having a lower abundance of core-fucosylated *N*-glycans (van de Bovenkamp et al. 2016). Yet, it is not clear why the number of antennae in IgG *N*-glycans seems to be limited to only two antennae (or three if the bisecting GlcNAc is considered also as an own branch). One possibility that may explain this is that antibody-producing plasma cells do not express the MGAT4 or MGAT5 enzymes needed for further branching. Another explanation could be that the addition of bisecting GlcNAc (or some other regulatory system) will prevent further branching of IgG *N*-glycans. The existence of such a system would be logical, given that an increase in *N*-glycan "bulkiness" brought about by additional branching might interfere with the folding and pairing of the Fc regions in the ER, and thereby alter its conformation known to be important for its binding to Fc receptors and antibody effector functions. Similarly, it is unclear why the Fab *N*-glycans display a higher proportion of more mature (more completely processed) *N*-glycans than those of Fc *N*-glycans. Whether this difference stems from better accessibility of the Fab glycans over Fc glycans, or something else such as increased extracellular glycosylation or decreased degradation of glycosidases, remains to be explored.

7.4 Golgi Microenvironment Is Important for Normal Processing and Maturation of *N*-Glycans

Despite the rather homogenous nature of high mannose type *N*-glycans arriving in the Golgi, *N*-glycans leaving the Golgi are much less so. For example, a single glycoprotein can carry complex, hybrid, and high-mannose *N*-glycans on the same polypeptide. Hybrid and complex type *N*-glycans can also display a variable number of antennae in their structure that may, or may not, carry sialic acid and/or fucose. While we do not have a clear picture at the molecular level of what determines the outcome in each case, this heterogeneity reflects both protein- and cell-specific processing of *N*-glycans brought about for example by epigenetic changes that determine what enzymes are expressed by the cell. Other factors that also modulate *N*-glycan biosynthesis are discussed below.

7.4.1 Golgi pH Homeostasis

The environmental cues outside or inside the cells can also contribute to *N*-glycan diversity. Unlike the ER, the other secretory pathway compartments, including the ER-Golgi intermediate compartment (ERGIC), the Golgi apparatus itself, and secretory vesicles, have uniquely acidic lumens, their pH decreasing along the pathway toward the plasma membrane (Paroutis et al. 2004). This pH gradient is crucial for their efficient functioning in membrane trafficking, glycosylation, proteolysis, protein sorting, or cargo transport (Kellokumpu 2019). Altered glycosylation due to abnormal Golgi pH is also responsible for several human disorders identified recently (Khosrowabadi and Kellokumpu 2020). Proper pH in the Golgi lumen appears to be important especially for the activity and assembly of the glycosyltransferase complexes in the Golgi. Previously, we have shown that the *trans*-Golgi β(1,4)GalT1 galactosyltransferase not only forms homomers in the ER but also heteromers with either ST3Gal3 or ST6Gal1 sialyltransferases upon its arrival in the Golgi (Hassinen et al. 2011; Hassinen and Kellokumpu 2014). Interestingly, these two heteromeric complexes assemble only in the acidic pH of the Golgi lumen (pH < 6.5). In the former case, complex formation could be prevented by increasing Golgi pH only by 0.2 pH units (Hassinen et al. 2011; Hassinen and Kellokumpu 2014). This increase was also sufficient to redirect the ST3Gal3 enzyme from the Golgi to post-Golgi compartments, consistent with their oligomerization-driven retention in the organelle (Rivinoja et al. 2009). The loss of the enzyme heteromers and enzyme mislocalization also coincided with reduced α(2,3)-sialylation and increased α(2,6)-sialylation of carcinoembryonic antigen (CEA) (Rivinoja et al. 2009). A similar decrease and increase in α(2,3)- and α(2,6)-sialylation, respectively, in CEA *N*-glycans has also been observed in cancer tissues in vivo (Kobata et al. 1995). Since Golgi resting pH is often elevated in cancer cells (Rivinoja et al. 2006), these findings suggest that Golgi resting pH may be used to regulate what linkage type will be used to link sialic acids to an *N*-glycan. This kind of switch from one linkage type to another one can have dramatic effects on cell behavior. For example, increased expression of α(2,6)-linked sialic acid in *N*-glycans can inhibit tumor cell apoptosis and activate growth factor pathways (Francisco et al. 2020; references therein).

Interestingly, the formation of the β(1,4)GalT1/ST6Gal heteromer was shown to increase markedly the catalytic activity of the β(1,4)GalT1 perhaps via substrate channeling (Hassinen et al. 2011). Alternatively, heteromer formation may also increase the accessibility of the donor or acceptor substrates to the active site of the β(1,4)GalT1, even though it is not directly involved in homodimer formation (Harrus et al. 2018). Yet, the active site is more exposed in the β(1,4)GalT1/ST6Gal heterodimers than it is in homodimers (Khoder-Agha et al. 2019b). In addition to 3D structures, this view is supported by the observation that a single mutation in the active site (H243) was able to abolish homodimer formation but not heterodimer formation.

Acidic Golgi resting pH is also needed the keep certain glycosyltransferases active. Accordingly, Golgi acidity (pH $<$ 6.5) is essential for the full catalytic activity of ST6Gal1 sialyltransferase but not for β(1,4)GalT1, nor the MGATs (Hassinen et al. 2011). Partly, this can be explained by the pH-sensitive interactions between the β(1,4)GalT1 and the two sialyltransferases acting on *N*-glycans (Hassinen and Kellokumpu 2014). Yet, it is likely that pH-dependent conformational changes in the tertiary structure of ST6Gal1 also contribute to the activity loss if the Golgi resting pH is close to neutral. Collectively, these data suggest that the main role of the decreasing pH gradient from the *cis*-to-*trans* side of the Golgi compartments (pH 6.7–pH 6.3) is to orchestrate mutual interactions between glycosyltransferases, to promote their active conformation, and to get them correctly localized, in accord with their suggested oligomerization-mediated retention in the Golgi (Nilsson et al. 2009).

7.4.2 Golgi Ion Homeostasis

Golgi lumen contains high amounts of calcium, magnesium, and manganese ions (Van Baelen et al. 2004; Pizzo et al. 2010; Vangheluwe et al. 2009). The presence of these divalent cations is important for cargo concentration and sorting (Chanat and Huttner 1991) as well as for glycosylation (Vanoevelen et al. 2007). The cations are transported into the Golgi lumen by the SERCA2 and SPCA1/2 type Ca^{2+}/Mn^{2+} pumps. Of these two, SERCA2 is enriched in the *cis*-Golgi, while SPCA1 is mainly present in the *trans*-Golgi (Vangheluwe et al. 2009). Unlike SERCAs, SPCAs are also engaged in Mn^{2+} transport (Vangheluwe et al. 2009; Wong et al. 2013). In addition to SPCAs, recent evidence suggests that TMEM165 mutations in patients cause a type II congenital disorder of glycosylation in humans by interfering with Mn^{2+} and Ca^{2+}/H^{+} transport (Dulary et al. 2017; Thines et al. 2018). Manganese is an essential trace metal and important co-factor needed for the catalytic activity of many inverting Golgi glycosyltransferases such as β(1,4)GalT1. The DXD motif typically present in these enzymes plays a key role in Mn^{2+}-mediated donor substrate (UDP-Gal) binding (Breton et al. 2005). Based upon the recent structure of the β(1,4)GalT1 homodimer (Harrus et al. 2018), Mn^{2+} appears to regulate transitions of the lid and the "Trp loop" that define the open (inactive) and closed (active) states of the enzyme. Accordingly, the Met340H mutant form of the enzyme that binds Mn^{2+} 25 times more avidly, blocks the β(1,4)GalT1 in the closed state, inactivates the enzyme, and prevents its ability to form homodimers.

7.4.3 Golgi Redox State

Reactive oxygen species and low oxygen tension (hypoxia) also contribute to Golgi glycosylation potential. Most often, their effects are mediated by hypoxia-inducible

factors (HIF1–3) that regulate the expression of a number of *N*-glycosylation-associated genes, including MGATs (MGAT2, MGAT-3, and MGAT5a and 5b), fucosyltransferases (FuT1, 2 and 7), sialyltransferases (ST3Gal1 and ST6Gal1) as well as nucleotide sugar transporters for UDP-galactose, CMP-sialic acid and UDP-*N*-acetylglycosamine (Koike et al. 2004; Shirato et al. 2010; Belo et al. 2015). Based upon these observations, Taniguchi et al. (Taniguchi et al. 2016) introduced the term "Glyco-redox" to link altered glycosylation with oxidative stress generated by hypoxia or reactive oxygen species (ROS). Their close association may also contribute to neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS). Hypoxia (or HIFs) may also induce cleavage of cell surface *N*-linked glycans and thereby affect cell–extracellular matrix interactions (Taniguchi et al. 2016; Eguchi et al. 2002, 2005). Oxidative stress and altered glycosylation have also been linked to high-fat diet, obesity, and the onset of type II diabetes mellitus (Ohtsubo and Marth 2006; Ohtsubo 2010; Ohtsubo et al. 2011). Marth and co-workers showed in their studies (Ohtsubo 2010; Ohtsubo et al. 2011) that high levels of free fatty acids inhibit the expression of MGAT4a, a glycosyltransferase needed for β(1,4)-GlcNAc branching of *N*-glycans as well as GLUT-2 glucose transporter in pancreatic β-cells. The β(1,4)-GlcNAc branch is normally required for cell surface localization of the glucose transporter, and thus for glucose transport into cells. Without the β(1,4)-GlcNAc branch, the GLUT-2 remains intracellular, leading to decreased glucose import, insulin export, and accumulation of glucose in the blood.

Recent evidence indicates that hypoxia can modulate *N*-glycosylation also in a HIF-independent manner via affecting the oxidative potential of the Golgi lumen (Hassinen et al. 2019). Surprisingly, in normoxic conditions, it is higher than that of the ER (the main site of disulfide bond formation in the cells). In hypoxic cells, however, Golgi oxidative potential equals that of the ER in normoxic cells. The cells also displayed less sialic acid in their cell surface *N*-glycans. Interestingly, this was shown to be associated with reduced formation of surface-exposed disulfide bonds in ST6Gal1 (and likely also in some other sialyltransferases including ST3Gal3), loss of its catalytic activity, and inability to interact with β(1,4)GalT1 (Hassinen et al. 2019). Therefore, the high oxidative potential in the Golgi lumen appears to be necessary for the catalytic activity of certain sialyltransferases. This "redox switch" guarantees that the ST6Gal1 remains inactive until it reaches the Golgi compartment where it is expected to function. Likewise, the β(1,4)GalT1 enzyme acquires full activity also in the acidic Golgi compartment after interacting with the ST6Gal1 sialyltransferase.

7.5 Concluding Remarks

N-glycosylation is a frequent and complex modification of proteins, and essential for both uni- and multicellular life. It regulates a plethora of cellular functions that range from protein folding, trafficking, sorting, localization, half-life, and signaling to

proliferation, migration, and adhesion with its surroundings. Therefore, it is also not surprising that we currently know a vast number of human disorders that are caused by, or are associated with, altered *N*-glycosylation. While previous work has provided us a clear overall picture of the basic principles in *N*-glycan biosynthesis, there is a big gap in our understanding of the factors that underlie cell-, tissue-, or organism-specific glycosylation patterns and their dynamic variability that starts during embryonic development continuing thereafter throughout our lives. We currently know that factors such as pH, redox potential, and changes in ion fluxes mainly in the Golgi compartment fundamentally affect and regulate the functioning and activity of glycosyltransferases expressed in a cell. Yet, there are many questions that remain unanswered. For example, how and why cells have evolved in such a complex way to make their *N*-glycans, needing removal of some sugar residues and replacing them with others instead of adding the right sugar in the beginning? Perhaps there is an evolutionary reason, as yeasts (an early eukaryote) produce mainly high mannose type *N*-glycans which needed to be modified to different ones in order to provoke immune responses only against them and other pathogens, and thereby survive. And what is the reason (or cause) of producing *N*-glycans which differ between two identical protein molecules? Does it result from a "sloppy" machinery that is prone to mistakes, or is there some purpose or benefit behind? Increasing biodiversity perhaps? Or is it a mark of ongoing evolution and trials to find the best fit for changing conditions? Is it regulated, or random? List is endless.

Nevertheless, these examples emphasize the need to understand in much more detail how glycans are made, how their synthesis is regulated and to what extent. An important issue also to keep in mind when one aims to produce optimally glycosylated antibodies for therapeutic use is to realize that yeasts, other lower eukaryotes or bacteria might not be the best choices to be used as hosts, as glycosylation is not just a simple outcome of enzymes present. It requires also conditions that support their full activity and complex mutual interplay necessary for their efficient functioning. Finally, we infer that there is an urgent need for developing more effective glycoengineering tools to edit glycans at will and thereby improve physicochemical and pharmacological properties of glycoprotein-based therapeutic compounds.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This chapter does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 8
Genetic Regulation of Immunoglobulin G Glycosylation

Azra Frkatovic, Olga O. Zaytseva, and Lucija Klaric

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Abstract Defining the genetic components that control glycosylation of the human immunoglobulin G (IgG) is an ongoing effort, which has so far been addressed by means of heritability, linkage and genome-wide association studies (GWAS). Unlike the synthesis of proteins, *N*-glycosylation biosynthesis is not a template-driven process, but rather a complex process regulated by both genetic and environmental factors. Current heritability studies have shown that while up to 75% of the variation in levels of some IgG glycan traits can be explained by genetics, some glycan traits are completely defined by environmental influences. Advances in both high-throughput genotyping and glycan quantification methods have enabled genome-wide association studies that are increasingly used to estimate associations of millions of single-nucleotide polymorphisms and glycosylation traits. Using this method, 18 genomic regions have so far been robustly associated with IgG *N*-glycosylation, discovering associations with genes encoding glycosyltransferases, but also transcription factors, co-factors, membrane transporters and other genes

A. Frkatovic · O. O. Zaytseva
Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia

L. Klaric (✉)
MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK
e-mail: lklaric@ed.ac.uk

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with no apparent role in IgG glycosylation. Further computational analyses have shown that IgG glycosylation is likely to be regulated through the expression of glycosyltransferases, but have also for the first time suggested which transcription factors are involved in the process. Moreover, it was also shown that IgG glycosylation and inflammatory diseases share common underlying causal genetic variants, suggesting that studying genetic regulation of IgG glycosylation helps not only to better understand this complex process but can also contribute to understanding why glycans are changed in disease. However, further studies are needed to unravel whether changes in IgG glycosylation are causing these diseases or the changes in the glycome are caused by the disease.

Keywords Immunoglobulin G · *N*-glycosylation · Genome-wide association study · Heritability

Abbreviations

CC	Collaborative Cross
CD4	Helper T-cells
CD8	Cytotoxic T-cells
CD14	Macrophages
CD15	Neutrophils
CD19	B-cells
DZ	Dizygotic
ER	Endoplasmic reticulum
GWAS	Genome-wide association study
HDL	High-density lipoprotein
IgG	Immunoglobulin G
LD	Linkage disequilibrium
MZ	Monozygotic
SNP	Single nucleotide polymorphism

8.1 Introduction

N-glycans attached to the immunoglobulin G (IgG) molecules influence the effector functions of IgG (Ferrara et al. 2011; Dekkers et al. 2017; Banda et al. 2008), reviewed in Gudelj et al. (2018), antibody half-life in the bloodstream, antigen-binding, auto-reactivity, and immune complex formation (van de Bovenkamp et al. 2016). Although *N*-glycan biosynthesis is, to a certain degree, stochastic and not template-driven, it is not a random assembly of all possible sugar residues. It is rather a complex but well-governed process that results in specific *N*-glycan profiles, characteristic for secreted IgG, produced by a specific cell type, namely, plasma cells. Defining the genetic component that controls this complex process is one of the

essential aims of glycobiology, which will provide new insights in the mechanisms of a number of pathologies where IgG is involved, as well as improve our understanding of humoral immune functions in general.

The biosynthesis of the conserved *N*-glycan core begins in the endoplasmic reticulum and continues through *cis*- and medial Golgi, while the majority of features characteristic for IgG glycans, like fucosylation and antennary modifications, are created in the *trans*-Golgi (Varki et al. 2017). Regulation of *N*-glycosylation is a complex process, dependent on the expression and activity of glycosyltransferases (GTs) (Klaric et al. 2020; Nairn et al. 2008), competition between GTs for their substrates, GT transport and localisation within Golgi, membrane properties, pH and ionic composition of Golgi apparatus (Kellokumpu 2019), availability and trafficking of sugar donors, as well as on the properties of the glycosylated protein, such as amino acid sequence and folding [reviewed in Pothukuchi et al. (2019)]. Naturally, expression and activity of all protein components involved in the glycosylation process are also controlled at transcriptional and post-transcriptional levels. Therefore, a diverse and complex network of interactions taking place on all possible cellular levels defines the types of *N*-glycans produced by a cell and attached to specific protein cargos. The resulting structural spectrum and corresponding quantities of glycans produced by a certain cell type or found on a certain glycoprotein are usually referred to as *N*-glycome.

The *N*-glycome of human IgG is distinct from those of other antibody classes (Clerc et al. 2016), and mainly includes complex-type biantennary *N*-glycans, with or without core fucose and bisecting *N*-acetylglucosamine (GlcNAc), with almost no highly-branched structures (Pučić et al. 2011). Both or only one antenna can be decorated with galactose residues, on top of which an *N*-acetylneuraminic acid residue can be added. A small amount of high-mannose *N*-glycans is also present in the IgG *N*-glycome. IgG *N*-glycosylation is known to be perturbed in diseases, ageing (Gudelj et al. 2018) and pregnancy (Bondt et al. 2014). Thus, one may regard these changing glycan features as potential biomarkers of health and age (Gudelj et al. 2018; Krištić et al. 2014; Vanhooren et al. 2007, 2009; Vilaj et al. 2019). A study of global variation of IgG glycome in human populations (Stambuk et al. 2020) showed that IgG galactosylation correlates with socioeconomic development indicators of the country of residence, probably reflecting exposure to pathogens, lifestyle, healthcare quality, and, perhaps, genetic differences between human populations. Evidence suggests that complex interactions between environment and genetic sequence have a vast impact on glycan biosynthesis, resulting in immediate glycan change or creating lasting modifications that are maintained through epigenetic mechanisms (Zoldos et al. 2012).

8.2 Heritability of the Human IgG *N*-Glycome

The main measurable parameter that gives us information on the relative contributions of the genes and environment to the variation of a certain phenotype—for instance, IgG *N*-glycome composition—is heritability. Heritability, in a broad sense,

is defined as the proportion of the observed phenotypic variance that can be attributed to variance of the genotype (Visscher et al. 2008). However, what the studies of heritability most commonly report, is the so-called "narrow-sense heritability", estimated from empirical data as a ratio of the variance of additive genetic effects to the total observed variance of a phenotype of interest (Visscher et al. 2008). Heritability estimates provide a better understanding of how exactly genes and environment shape a certain phenotype, allow to make meaningful comparisons between populations and are essential prerequisites for gene-mapping studies that can pinpoint genes responsible for the phenotype of interest and discover new biomarkers of disease. There are multiple technical approaches to heritability estimation, and the choice of a particular method depends on the available observations and the purpose of the study (Visscher et al. 2008). One of the earliest analyses of IgG *N*-glycome heritability was performed in a cohort of 906 individuals from a Croatian island of Vis using genealogical information (Pučić et al. 2011). In general, heritability of *N*-glycan levels varied from insignificant to over 50% in the case of sialylation-related traits (Fig. 8.1). This approach to estimating heritability, however, does not account for the possible contribution of a shared environment between related individuals, and therefore, some of the heritability estimates might be inflated.

A classic approach to study relative contributions of genetic and environmental factors to human traits is twin studies (Tan et al. 2015). Usually, this kind of

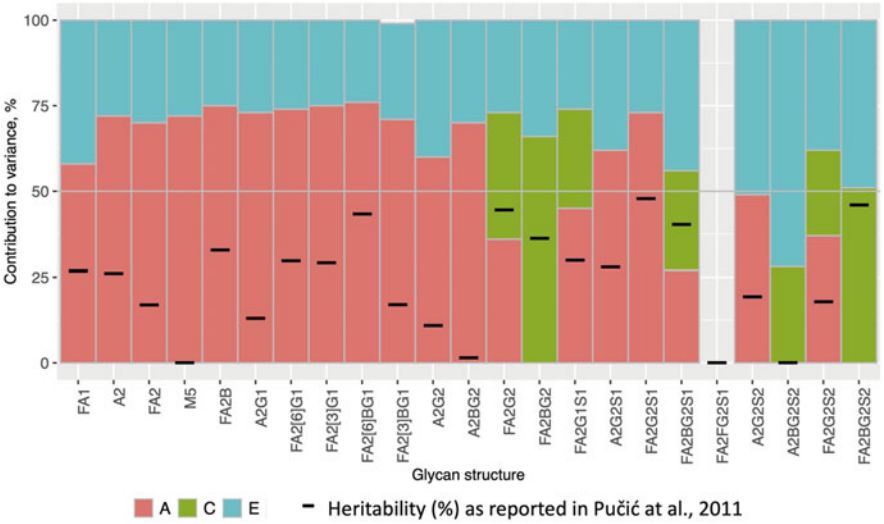

Fig. 8.1 Heritability estimates and partitioning of variance for IgG *N*-glycans based on data published in Pučić et al. (2011) and Menni et al. (2013). Variance components, as estimated by Menni et al using twins study approach: A, additive genetic variance (heritability); C, common environment variance; E, unique environment variance. X-axis: IgG glycosylation traits; y-axis: contributions of A, C, and E components to the total variance of glycosylation traits (%). Black lines represent heritability estimates by Pučić et al. based on pedigrees (genealogical information)

methodology employs a cohort of di- (DZ) and monozygotic (MZ) twins. MZ twins are genetically identical and DZ twins share approximately a half of their genes, so when a phenotype has significantly higher concordance in MZ twins, than in DZ, we can assume that this phenotype is largely influenced by the genes. At the same time, both DZ and MZ twins are exposed to similar environments, including uterine environment, parenting, education, family lifestyle, and quality of life, so the heritability estimates based on twin studies can also be somewhat inflated. However, when a phenotype has a similar concordance rate between MZ and DZ twins, it is supposed to be under the influence of the environmental factors shared by the twins. If a phenotype is largely discordant between both MZ and DZ twins, it is assumed to be controlled by environmental factors that are unique for an individual, for instance, personal lifestyle, life choices, accidents, etc. Modern methods of mathematical modelling using maximum likelihood approaches allow to decompose the variance observed in a quantitative trait measured in MZ and DZ twins into genetic, shared, and unique environmental components (Tan et al. 2015). Such an approach was applied to study the heritability of IgG *N*-glycosylation features (Menni et al. 2013). In this study, *N*-glycans were enzymatically released from the total pool of IgG isolated from plasma of 220 MZ and 310 DZ twin pairs and chromatographically separated into 24 peaks, each containing one major *N*-glycan structure. Heritability was estimated for relative abundances of 22 individual *N*-glycan structures found on IgG and for 54 derived traits that describe relative abundances of *N*-glycans that share structural features (e.g. all glycans with core fucose). For more than half of all *N*-glycan traits studied, 51 of the 76, at least 50% of the observed variance was explained by genetic factors. In total, only three individual glycans and four derived traits were not influenced by genetic factors at all. It is difficult to directly compare the results of this study with heritability estimates based on genealogy (Pučić et al. 2011). However, one can note that some of the sialylation traits, that were found to be highly heritable in the genealogical study, were not as much controlled by genetics according to the twin analysis and instead were under the considerable influence of the shared environment, which was not accounted for in Pučić et al. (2011). Relative abundances of most neutral glycan structures were found to be highly heritable (heritability >50%) and almost not affected by shared environment, while for individual sialylated structures the degree of estimated heritability varied from 0 to over 70% (Fig. 8.1).

Such discrepancies between heritability estimates of the same phenotype in different studies are not uncommon and are bound to happen when heritability is estimated with different methods in different populations (Tenesa and Haley 2013). One might ask a logical question—which estimate is then closer to the truth? Unfortunately, there is no clear answer to such a question. Estimated heritability of the same phenotypic trait can differ between populations because of the differences in frequencies of certain mutations, or because variability of a trait changes depending on the environment. An interaction between genes and environment is an important factor when the studied phenotype is concerned, so that in particular environments a phenotype may become more or less heritable (Visscher et al. 2008). It is widely accepted that even though it is hard to generalise heritability

estimates obtained in a single study (Tenesa and Haley 2013; Moore and Shenk 2017), nevertheless, the estimates obtained in different studies give a researcher a general idea of how relatively important are nature and nurture for the studied trait. For instance, in the case of IgG *N*-glycan traits, the findings published in Pučić et al. (2011) and Menni et al. (2013) can provide the lower and the upper limits of possible heritability of *N*-glycan abundances in the IgG *N*-glycome.

However, these methods give no information on what kind of genes and how many of them drive the observed variability of the IgG *N*-glycome composition. This question can be answered by the means of genetic association studies that are specifically designed to test if a specific region of a chromosome or a specific mutation in the genome could be controlling a certain trait.

8.3 Linkage Studies of Mouse *N*-glycome

Genetic association studies are performed to find out if a certain genetic locus could be influencing a phenotype of interest. This kind of study uses the information on how common polymorphisms, such as point mutations, insertions, and deletions are distributed in the population. The linkage disequilibrium (LD) mapping approach uses the fact that some groups of genetic variants situated within the same recombination block on a chromosome are more likely to be inherited together. Such groups of alleles are called haplotypes. By testing in a population whether a qualitative phenotype or the value of a quantitative phenotype, is correlated with the presence of a certain known haplotype, linkage analysis can identify chromosomal regions harbouring the genes influencing the trait of interest. It is assumed, that even if a causal mutation is not genotyped in the studied population, it will be tagged by some common genetic variants in LD. Further inspection of the identified locus can reveal the truly causal variants influencing the studied phenotype.

A linkage study directed at IgG *N*-glycosylation regulation was performed in the Collaborative Cross (CC) mouse cohort, by quantifying IgG *N*-glycans in 589 mice of 95 inbred strains (Krištić et al. 2018). The CC resource was specifically developed for mapping genes that underlie complex phenotypes in mice, commonly used as model organisms (Morahan et al. 2008). It provides a population of inbred mouse strains, which are derived from crosses of eight founder strains. Thus, the genomes of the CC inbred strains result from recombination of the eight founder genomes, and each strain has a unique mosaic pattern of inheritance of the genetic variants originating from the founders. The position of chromosomal blocks inherited from any of the eight founders in each of the CC strains was defined by genotyping. By correlating abundances of certain *N*-glycans in the IgG *N*-glycomes with the presence of genome regions derived from certain founder strains one can tell which parts of the mouse genome are harbouring candidate genetic variants that affect IgG *N*-glycosylation. Krištić et al. identified at least five genetic regions in the mouse genome that are potentially involved in the regulation of 11 individual *N*-glycan structures. The sizes of the chromosomal blocks inherited from founder strains in

this case spanned several megabases and could contain dozens of genes and their promoter regions, which made prioritisation of candidate genes an important task. Krištić et al. focused primarily on the genes in the loci of interest that contained missense mutations specific for the founder alleles that correlated with differing abundances of glycan structures. For instance, the levels of bisected *N*-glycans were associated with missense genetic variants in the *Mgat3* gene, encoding β-1,4-mannosyl-glycoprotein-4-β-*N*-acetylglucosaminyltransferase (GnT-III or MGAT3) (Taniguchi et al. 2014), an enzyme responsible for the addition of bisecting GlcNAc. In general, the percentage of bisected structures in the IgG *N*-glycome of most commonly used laboratory mouse strains is rather low, around 1–3% (de Haan et al. 2017; Zaytseva et al. 2018), so that in some studies it was not even reported (Blomme et al. 2011). However, CC mice that inherited an *Mgat3* allele from either of the two outbred CC founder strains, CAST or PWK, exhibited a significantly higher incidence of bisection in their IgG *N*-glycomes. Other proposed candidate genes, supposedly affecting digalactosylated and monosialylated glycans, were *Ighg1*, *Ighg2*, and *Ighg2c*, encoding the constant regions of immunoglobulin heavy gamma chains 1, 2b, and 2c, respectively. These genes are a part of a cluster of tightly linked genes, coding for constant regions of Ig heavy chains and are mainly inherited together, so it was not possible to attribute the differences in IgG *N*-glycosylation to any of these genes specifically. Studies of IgG subclass-specific *N*-glycomes, however, show that a relatively rare IgG1 allotype encoded by an *Ighg1* allele found in C57BL/6, CD1 and NOD mice is characterised by lower sialylation and galactosylation of *N*-glycans attached to the CH2 domain of IgG. This finding is in line with the studies showing that point mutations leading to amino acid substitutions in the heavy chain of IgG can result in alterations of IgG *N*-glycome (Lund et al. 1996; Rose et al. 2013).

8.4 Genome-Wide Association Studies of Human *N*-Glycome

Linkage analyses are a great tool for mapping which genomic loci have an effect on a phenotype, but suffer from a low mapping resolution—given the low number of recombination events per meiosis, associated regions covered many genes, requiring further analyses to pinpoint the causal gene. At the beginning of the twenty-first century, the Human Genome Project (Lander et al. 2001) released the first sequence of the human genome, which, together with the quantification of LD structure from the HapMap project (Belmont et al. 2005), facilitated the development of dense commercial Single Nucleotide Polymorphism (SNP) arrays. These arrays captured most of the common genetic variation and enabled affordable genotyping of hundreds of thousands of SNPs in thousands of individuals and were a start of the genome-wide association study era. Genome-wide association studies (GWAS) are high-resolution association scans across the whole genome, where each SNP in the

genome is tested for association with a phenotype. As a result, these analyses "zoom in" from associations with several megabases long regions in the linkage studies, to individual SNPs. The availability of reliable high-throughput methods for glycan analysis has enabled GWAS to help further elucidate the genetic background of IgG glycosylation by testing the association of the glycan levels with SNPs measured in a large group of individuals.

The first GWAS of IgG *N*-glycome was performed by Lauc et al. (2013), testing for genome-wide associations with 77 IgG *N*-glycome traits measured by ultra-performance liquid chromatography (UPLC) in 2247 individuals of European descent. Nine genomic regions (also called loci) were found to be significant on the genome-wide level (association *p*-value $\leq 5 \times 10^{-8}$). Four of these regions contained genes encoding glycosyltransferases (*ST6GAL1*, *B4GALT1*, *MGAT3*, *FUT8*)—enzymes catalysing the addition of sugar units to the growing glycan chain. Although not having an apparent role in glycosylation, five additional loci were discovered containing the following genes: *IKZF1*, *IL6ST-ANKRD55*, *SUV420H1*, *ABCF2-SMARCD3* and *SMARCB1-DERL3*. Statistically significant associations of glycan traits with variants at glycosyltransferase loci gave the first indication that GWA study for IgG glycans could identify genes relevant for glycosylation, as well as identify novel genes that have a potentially important, yet unknown role in this process.

The next attempt to discover additional genomic loci associated with IgG glycosylation applied a multivariate approach to GWAS of 23 directly measured IgG *N*-glycosylation phenotypes measured by UPLC in the ORCADES (Orkney Complex Disease Study; $N = 1960$) (Shen et al. 2017). In contrast to the more commonly used univariate approach, where an SNP is associated with one phenotype at a time, multivariate approaches jointly analyse the association of multiple phenotypes with an SNP (Solovieff et al. 2013). This approach takes into account the correlation structure among glycan traits, which might be due to shared genetic regulation, and provides greater power to detect associations (Solovieff et al. 2013), resulting in the discovery of more associated loci compared to a univariate study of the same sample size. In Shen et al., individual *N*-glycan traits were grouped to describe certain features of IgG *N*-glycome, such as fucosylation, bisection, galactosylation, etc., and association tests were performed jointly using all glycan traits from a specific group. This resulted in replication of five loci previously identified in Lauc et al. (2013), but also in the discovery of five novel loci: *IGH*, *ELL2*, *HLA-B-C*, *FUT6-FUT3*, *AZI1*, *and TMEM105* (Shen et al. 2017). These newly discovered loci were not detected in the univariate analysis, further confirming and addressing the need for multivariate approaches in complex trait analysis.

In contrast to the GWA studies by Lauc et al. (2013) and Shen et al. (2017), the GWAS by Wahl et al. (2018) was performed on liquid chromatography-electrospray mass spectrometry (LC–ESI-MS) measurements in KORA F4 (Cooperative Health Research in the Region of Augsburg; $N = 1836$). LC-ESI-MS glycoprofiling provides glycan measurements specific for IgG subclasses. With the new data type, the study replicated six of the previously known loci and discovered one novel locus on chromosome 1, containing the *RUNX3* gene, but, more importantly, highlighted that

the major difference between genetic regulation of *N*-glycomes of different IgG subclasses lies in the regulation of bisection and fucosylation.

The outcomes of these GWA studies indicated the polygenic nature of IgG *N*-glycosylation, where apart from glycosyltransferase genes that have a big effect on glycosylation, many other genes with smaller effects are also involved in the process. This, in turn, suggests that many associated genes cannot be detected in small sample sizes due to low statistical power. Therefore, a GWAS of 77 IgG *N*-glycan traits measured by UPLC was performed by combining summary statistics from four European cohorts with a total sample size of 8090 (Klaric et al. 2020). As the result of this genome-wide meta-analysis, 13 of the previously known loci were replicated and 14 novel loci were identified. Since genomic regions associated with glycan traits can span multiple genes, it is often a challenge to point to the actual causal genes involved in IgG glycosylation. While the previous studies focused mainly on the pre-existing knowledge on biological functions of the genes to suggest the most plausible candidate, this study applied several computational approaches to refine the list of IgG glycosylation-related genes. For obvious reasons, candidate genes that are known to be involved in protein glycosylation, such as enzymes directly involved in the process, were prioritised first. Next were genes with non-synonymous variants associated with IgG *N*-glycosylation since implied changes in amino acid sequence could potentially impact protein structure and function, thus influencing IgG glycosylation. Genetic variants can often affect multiple traits, a phenomenon known as pleiotropy. In the next prioritisation approach, the candidate variants were tested for pleiotropy with gene expression, i.e. if the same variant could be affecting both *N*-glycosylation of IgG and expression of any genes in the tissues relevant for IgG production or functions. Last but not least, candidate associations were subjected to pathway enrichment analysis (also referred to in this text as gene-set enrichment analyses). This type of gene prioritisation uses the information on which genes belong to certain biological pathways based on their co-expression in different cells and tissues or/and on well-known and characterised biological pathways. Then the method allows to test if the current list of genes is statistically significantly overrepresented in some specific pathways and decide if certain candidate genes are more relevant to the *N*-glycosylation of IgG than the others. When no other evidence was available for any of the genes in the locus, the gene closest to the strongest association signal was reported (also called positional mapping). The overall results of these prioritisation efforts can be seen in Fig. 8.2, while Table 8.1 lists gene associations for each glycan trait. Details for gene-prioritisation for each genomic locus, organised by chromosome, can be seen in Fig. 8.2 and paragraphs that follow.

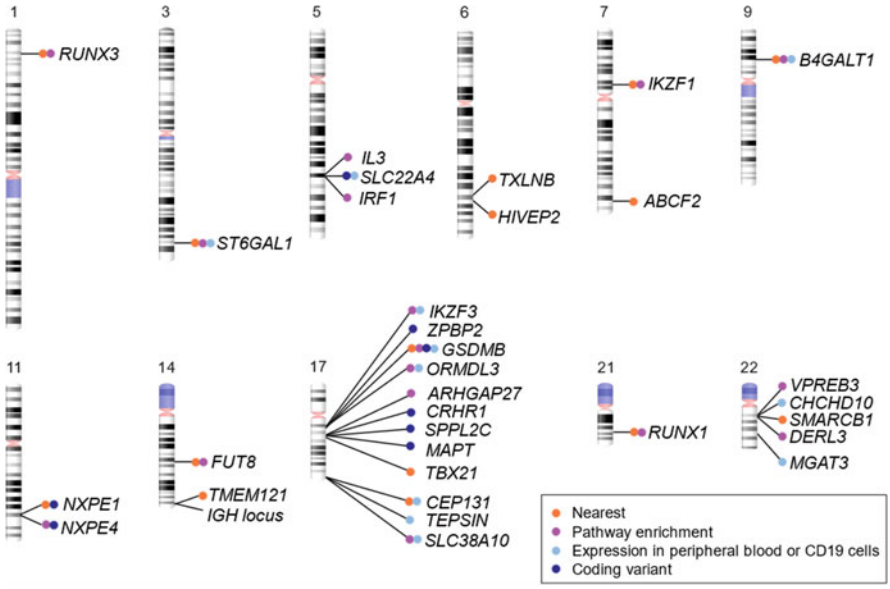

Fig. 8.2 Chromosomal location of currently known associations with IgG glycosylation across human genome. The annotated genes were prioritised based on the approaches labelled with coloured dots: being the nearest to the strongest association in the region (orange), pathway enrichment (purple), pleiotropy with gene expression in peripheral blood or CD19 B cells (light blue), and presence of missense mutations (dark blue)

8.4.1 Genomic Loci Associated with IgG N-*Glycosylation*

8.4.1.1 Chromosome 1

The locus on chromosome 1 identified in GWAS by Wahl et al. (2018) and later replicated in Klaric et al. (2020) contains a single gene *runt-related transcription factor 3* (*RUNX3*), which codes for a transcription factor with an important role in a range of developmental processes including haematopoiesis (Voon et al. 2015). Evidence suggests that *RUNX3* might play a role in B cell maturation (Whiteman and Farrell 2006) and T cell differentiation, particularly into CD4+ helper and CD8+ cytotoxic cells (Overgaard et al. 2015; Steinke et al. 2014). Previously, it was shown that IgG1 glycosylation depends on B cell stimuli, including T cell-derived cytokines and metabolites (Wang et al. 2011), hence Wahl et al. (2018) suggested that *RUNX3* could indirectly affect the glycosylation of antibodies by influencing T cell differentiation.

Table 8.1 Detailed list of associations of IgG glycans and loci across human genome based on Klaric et al. (2020)

Genomic Locus	Prioritised Gene	 A2	FA2	FA2B	A2G1	FA2[6]G1	FA2[3]G1	FA2[6]BG1	FA2[3]BG1	A2G2	A2BG2	FA2G2	FA2BG2	FA2G1S1	A2G2S1	FA2G2S1	FA2BG2S1	FA2FG2S1	A2BG2S2	FA2G2S2	FA2BG2S2	FBG2*/FG2*	FG2S1/(FG2+FG2S1+FG2S2)
1:25226001-25345011	*RUNX3*																					■	
3:186712711-186738421	*ST6GAL1*						■				■	■	■	■		■			■	■	■		
5:131026218-131833599	*IRF1; IL3, SLC22A4*	■																					
6:139617590-139636003	*TXLNB*							■	■				■										
6:143150223-143203591	*HIVEP2*											■											
7:50325717-50361683	*IKZF1*	■			■			■		■	■								■				
7:150906453-150969535	*ABCF2*	■		■	■									■									
9:33041761-33186080	*B4GALT1*		■				■					■				■				■			
11:114323627-114450529	*NXPE1; NXPE4*																						■
14:65472891-66284991	*FUT8*	■			■					■					■				■				
14:105966019-106002352	*TMEM121; IGH*					■	■							■									
17:37903731-38112190	*ORMDL3; GSDMB; IKZF3; ZPBP2*	■			■																		
17:43463492-44896083	*CRHR1; SPPL2C; MAPT; ARHGAP27*												■										
17:45518583-45874272	*TBX21*				■					■													
17:79165171-79257880	*SLC38A10; CEP131; TEPSIN*												■			■							
21:36546756-36665202	*RUNX1*			■																			
22:24093789-24182500	*SMARCB1; CHCHD10; YPREB3*			■				■	■				■										
22:39774448-39860868	*MGAT3*			■				■	■				■				■			■			

8.4.1.2 Chromosome 3

The strongest association from all four conducted GWA studies was observed in the region on chromosome 3 harbouring *β-galactoside-α-2,6-sialyltransferase 1* (*ST6GAL1*) gene. It codes for a sialyltransferase involved in the addition of α2,6-linked sialic acid residues to Galβ1,4-GlcNAc structures in *N*-linked glycans of glycoproteins (Weinstein et al. 1982; Dall'Olio 2000), thus making it the most plausible candidate gene in the region.

8.4.1.3 Chromosome 5

One of the genomic regions on chromosome 5 associated with the percentage of agalactosylated (G0) glycans in total IgG glycans (Klaric et al. 2020) spans 800 kb and 11 genes: *SLC22A4*, *IRF1*, *IL3*, *SLC22A5*, *CSF2*, *FNIP1*, *P4HA2*, *P4HA2-AS1*, *MEIKIN*, *PDLIM4*, and *ACSL6*. In this locus, gene-set enrichment analysis prioritised *interferon regulatory factor 1* (*IRF1*) and *interleukin 3* (*IL3*) genes. *IRF1* gene codes for a transcriptional regulator of T cell differentiation and is involved in cell proliferation, DNA damage response and apoptosis, as well as the activation of genes in innate and adaptive immune response (Oshima et al. 2004; Fragale et al. 2008). *IL3* gene codes for interleukin 3, a cytokine with an important function in the differentiation and proliferation of haematopoietic and lymphoid cell lineages. Pleiotropy analysis between gene expression and IgG glycosylation also suggested *solute carrier family 22 member 4* (*SLC22A4*), gene encoding a sodium-ion dependent plasma membrane transporter of organic cations (Tokuhiro et al. 2003) as a candidate gene in this locus. In addition, glycosylation-associated variant in *SLC22A4* is a missense variant resulting in leucine to phenylalanine change (rs1050152:Leu>Phe) but was predicted to have no influence on the protein structure. However, obtained results and previous indications for functions of *SLC22A4*, *IRF1* and *IL3* genes could not provide sufficient evidence to prioritise one gene over the others in the given locus.

8.4.1.4 Chromosome 6

The first locus on chromosome 6 contains a single gene, *taxilin beta* (*TXLNB*). *TXLNB* gene is expressed in skeletal muscle and heart tissues and has a role in myogenesis (Sakane et al. 2016). No additional evidence other than positional mapping was found to explain and support the relationship of this gene with IgG glycosylation events.

Another significant association was found in the locus harbouring *human immunodeficiency virus type I enhancer-binding protein 2* (*HIVEP2*) gene, which encodes a transcription factor involved in the regulation of gene expression during brain development (Takagi et al. 2006). *HIVEP2* knock-out in mice has shown to

upregulate the gene expression of nuclear factor kappa-light-chain-enhancer of activated B Cells (NF-κB) and cause inflammation in several brain areas, implicating a likely causal relationship between immune response and neurodevelopmental disorders (Takao et al. 2013; Choi et al. 2015).

8.4.1.5 Chromosome 7

A genomic region on chromosome 7 harbouring *IKAROS family zinc finger 1* (*IKZF1*) gene was among the five discovered loci by Lauc et al. (2013), with no previously implicated roles in protein glycosylation. The association with this region was later replicated both by Wahl et al. (2018) and Klaric et al. (2020). *IKZF1* gene codes for DNA-binding protein Ikaros, responsible for the regulation of lymphocyte differentiation and has been previously implicated to influence effector pathways in the humoral immune response by controlling class switching recombination (Sellars et al. 2009). As shown in Lauc et al. (2013), the *IKZF1* knock-out mice exhibit somewhat lower levels of core fucosylation and higher levels of glycans with bisecting GlcNAc, implying that *IKZF1* is potentially involved in the regulation of fucosylation by promoting the addition of bisecting GlcNAc (Lauc et al. 2013). Klaric et al. further hypothesised that *IKZF1* and *IKAROS family zinc finger 3* (*IKZF3*) genes play a role in regulation of expression of *fucosyltransferase 8* (*FUT8*) gene and performed a functional validation in a B cell derived lymphoblastoid cell line, MATAT6. Knock-down of *IKZF1* resulted in downregulated expression of *IKZF3* gene and significant up-regulation of *FUT8* expression, thus resulting in increased levels of fucosylated structures (Klaric et al. 2020). The experiment provided direct evidence of *IKZF1* role in regulation of *FUT8* gene expression and regulation of IgG glycosylation.

The region on chromosome 7 containing *SWI/SNF-related*, *matrix-associated*, *actin-dependent regulator of chromatin*, *subfamily D*, *member 3* (*SMARCD3*) and *ATP binding cassette subfamily F member 2* (*ABCF2*) genes was also indicated as genome-wide significant in GWAS by Lauc et al. (2013) and replicated in a subsequent study by Klaric et al. (2020). The *ABCF2* gene codes for the ATP-binding cassette, sub-family F, member 2 protein which has a potential function in transmembrane transport of molecules (Liu et al. 2016). *SMARCD3* is involved in the SWI/SNF chromatin remodelling complex which is likely to regulate transcription of genes by altering the chromatin structure and exposing genes regulatory regions to transcriptional machinery (Wang et al. 1996). Expression of *ABCF2* was detected in both peripheral blood and immune cells, as well as expression of *SMARCD3* in the CD15 neutrophil cells, but there was no evidence for pleiotropy with IgG *N*-glycosylation (Klaric et al. 2020).

8.4.1.6 Chromosome 9

The genomic locus identified on chromosome 9 contains *beta-1,4-galactosyltransferase 1* (B4GALT1), gene coding for an enzyme catalysing the transfer of galactose from UDP-galactose to the GlcNAc residue in the nonreducing end of N-linked glycans, resulting in the disaccharide moiety LacNAc with a β1–4-glycosidic linkage (Hennet 2002). In a study by Lauc et al., variants from this region were mainly associated with differences in sialylation and percentage of bisection rather than galactosylation measures (Lauc et al. 2013). However, associations with these traits were still considered plausible since galactosylation is a prerequisite for the addition of a sialic acid residue, further supported by the evidence that enzymes adding galactose and bisecting GlcNAc units compete for the same substrate (Fukuta et al. 2000). In Klaric et al., *B4GALT1* was linked to immune system processes and variants identified in the locus were pleiotropic with gene expression of *B4GALT1* in CD4 helper T cells and CD19 B cells (Klaric et al. 2020), suggesting that expression of this gene in relevant cells influences IgG glycosylation.

8.4.1.7 Chromosome 11

A region on chromosome 11 contains two genes encoding members of neurexophilin and PC-esterase domain family: *NXPE1* and *NXPE4*. The role of *NXPE4* was indicated in regulation of colorectal cancer development and progression, likely functioning as a tumour suppressor (Liu et al. 2019). Both genes contain a missense variant associated with IgG glycans but given the weak LD between the two SNPs, it might be an indication for two separate association signals, each affecting a different set of IgG glycan traits. While no evidence of pleiotropy with gene expression was found, *NXPE4* was prioritised for being a member of pathways enriched for IgG glycosylation genes (Klaric et al. 2020).

8.4.1.8 Chromosome 14

Associations with variants in the region on chromosome 14, harbouring *FUT8* gene were discovered and replicated in all four GWA studies (Klaric et al. 2020; Lauc et al. 2013; Shen et al. 2017; Wahl et al. 2018). *FUT8* gene codes for fucosyltransferase 8, an enzyme responsible for the transfer of fucose from GDP-fucose to the innermost GlcNAc residue of *N*-linked glycans, resulting in α1,6-fucose residue (Taniguchi et al. 2014). Previously described and well-understood function of *FUT8* explains its involvement in IgG glycosylation and clearly points to *FUT8* as the causal gene in the region. It is interesting to note that all associations with this locus are with afucosylated glycans (Table 8.1). While perhaps contra intuitive, this might suggest that availability of the substrate (afucosylated

glycan) is a rate-limiting step in fucosylation from the perspective of genetic regulation.

The second region on chromosome 14, discovered both in the multivariate IgG *N*-glycome GWAS (Shen et al. 2017) and Klaric et al. (2020) contains several genes. *Transmembrane protein 121* (*TMEM121*) has no function described to date, while *immunoglobulin heavy locus* (*IGH*) spans genes encoding heavy chains of immunoglobulins, including IgG heavy chain genes (*IGHG*). Although the direct relationship between *IGH* locus and glycosylation is not yet understood, previous research showed that the amino-acid sequence of the constant region of the IgG heavy chain has an impact on the *N*-glycome composition (Lund et al. 1996; Rose et al. 2013). In addition, a QTL study of IgG *N*-glycome regulation in mice proposed genes coding for the constant region of IgG heavy chain as candidate genes influencing sialylation and galactosylation of IgG (Krištić et al. 2018).

8.4.1.9 Chromosome 17

The first locus on chromosome 17 harbours four genes: *ORMDL sphingolipid biosynthesis regulator 3* (*ORMDL3*), *gasdermin B* (*GSDMB*), *IKAROS family zinc finger 3* (*IKZF3*) and *zona pellucida binding protein 2* (*ZPBP2*) (Klaric et al. 2020). Both *GSDMB* and *ZPBP2* genes contain missense SNPs, rs2305479, resulting in a potentially damaging change from glycine to arginine in GSDMB, and rs11557467, resulting in a tolerated change from serine to isoleucine in ZPBP2. Pleiotropy between glycosylation and gene expression was observed for *GSDMB* and *ORMDL3 genes* in CD19 B cells, *ORMDL3* in CD4 helper and CD8 cytotoxic T cells and expression of *IKZF3* in peripheral blood. All three genes, *GSDMB*, *ORMDL3* and *IKZF3*, were also prioritised by the pathway enrichment analysis, making them all potential gene candidates in the region (Klaric et al. 2020). GSDMB is a member of a protein family likely having a role in pyroptosis (highly inflammatory form of programmed cell death), thereby triggering a strong inflammatory response (Ding et al. 2016). *ORMDL3* gene was shown to modulate T-lymphocyte activation via controlling the influx of calcium ions in the endoplasmic reticulum upon antigen binding (Carreras-Sureda et al. 2013). IKZF3 is a member of a family of haematopoietic-specific transcription factors that play a critical role in regulating B cell and T cell development (Rebollo and Schmitt 2003). In addition, *ORMDL3-GSDMB-IKZF3-ZPBP2* region has previously been identified as a susceptibility locus for childhood-onset asthma (Moffatt et al. 2007), inflammatory bowel disease (Anderson et al. 2011), type 1 diabetes (Saleh et al. 2011), and rheumatoid arthritis (Stahl et al. 2010), suggesting that future insights into the role of these genes in IgG glycosylation could also increase our understanding of the disease development and progression.

The second region on chromosome 17 spans 1.5 Mb and harbours 12 genes, for some of which evidence for prioritisation was found. Pathway enrichment analysis prioritised *Rho GTPase activating protein 27* (*ARHGAP27*) (Klaric et al. 2020), a gene that encodes a member of a protein family that activates Rho-type GTP

metabolising enzymes and whose function is implicated in mitogen-activated protein kinase signalling (Julià et al. 2018). Three genes, *corticotropin-releasing hormone receptor 1* (*CRHR1*), *signal peptide peptidase like 2C* (*SPPL2C*) and *microtubule-associated protein tau* (*MAPT*), contain a non-synonymous variant associated with glycosylation (rs16940665, stop lost variant in *CRHR1*; rs12185233, potentially damaging variant resulting in a change from Arginine to Proline in *SPPL2C*; rs754512, stop gained variant in *MAPT*) (Klaric et al. 2020). *CRHR1* gene encodes a receptor that binds the corticotrophin-releasing hormone family, which plays various roles in stress response but also in inflammatory processes (Zhu et al. 2011). Intramembrane-cleaving aspartic protease encoded by the *SPPL2C* gene was shown to participate in vesicular transport and possibly cause retention of cargo proteins in the ER, thereby affecting cellular processes, such as protein glycosylation, through miss-localisation of glycan-modifying enzymes (Papadopoulou et al. 2019). *MAPT* gene codes for the tau protein which is expressed throughout the central nervous system and was previously implicated in neurodegeneration (Strang et al. 2019). Strong LD in the region and lack of evidence for pleiotropy with gene expression make it hard to point to a single gene candidate.

T-Box 21 (*TBX21*) gene is located closest to the strongest signal in the third region on chromosome 17 (Klaric et al. 2020). TBX21 is a member of the T-box family of transcription factors expressed in multiple cells of the adaptive and innate immune system, with an important role in development, survival, and activation of the cells in immune response (Lazarevic et al. 2013). The region contains expression quantitative trait loci (eQTL) weakly associated with the expression of *TBX21* in CD4 helper T cells (Klaric et al. 2020). Additional four genes can be found in the region: *EF-hand calcium-binding domain 13* (*EFCAB13*), *aminopeptidase puromycin sensitive* (*NPEPPS*), *karyopherin subunit beta 1* (*KPNB1*), and *TBK1 binding protein 1* (*TBKBP1*). However, insufficient evidence for their prioritisation positions *TBX21* as the top gene candidate for this locus.

Four genes, *NADH:ubiquinone oxidoreductase complex assembly factor 8* (*NDUFAF8*), *solute carrier family 38 member 10* (*SLC38A10*), *adaptor related protein complex 4 accessory protein* (*TEPSIN*) and *centrosomal protein 131* (*CEP131*), are found in the locus on chromosome 17 first discovered by Shen et al. (2017) and replicated in Klaric et al. (2020). Evidence for pleiotropy of gene expression and IgG glycosylation was found for the following: expression of *SLC38A10* in macrophages, expression of *TEPSIN* in neutrophils and peripheral blood, and expression of *CEP131* in peripheral blood (Klaric et al. 2020). *CEP131* gene is involved in the formation of cilia and flagellum but is also shown to be an important regulator of genome stability (Hall et al. 2013; Staples et al. 2012). *CEP131* gene is located closest to the strongest association in the region, while pathway enrichment analysis prioritised *SLC38A10* (Klaric et al. 2020). *SLC38A10* gene encodes a member of the solute carrier (SLC) family-38 of transporters with a suggested role in signalling pathways regulating protein synthesis (Tripathi et al. 2019). Given that none of the genes in the region have functions linking them to glycosylation or immunity in general, all of them still remain biologically plausible target genes for IgG *N*-glycosylation.

8.4.1.10 Chromosome 21

The region on chromosome 21 is located downstream from *runt-related transcription factor 1* (*RUNX1*) gene, which codes for a transcription factor important for embryonic development, tumorigenesis, and inflammatory response (Scheitz and Tumbar 2013) and with a crucial role in haematopoiesis (Okuda et al. 1996). Additionally, due to existing evidence of interaction with RUNX3 (Spender et al. 2005) and role in pathways enriched for IgG glycosylation, it is considered a biologically plausible candidate.

8.4.1.11 Chromosome 22

SWI/SNF related, *matrix associated*, *actin-dependent regulator of chromatin*, *subfamily B*, *member 1* (*SMARCB1*) is one of six genes found in a locus on chromosome 22. *SMARCB1* gene encodes a core subunit of the SWI/sucrose non-fermenting ATP-dependent chromatin remodelling complex (SWI/SNF), which plays a key role in the regulation of gene transcription (Kalimuthu and Chetty 2016). *SMARCB1* also has an immune system-related function as its role was described in inhibition of antiviral activity, neurodevelopment, tumour formation, cell differentiation, and proliferation (Pottier et al. 2007). Another gene in the region, *derlin 3* (*DERL3*), codes for a component of endoplasmic reticulum-associated degradation for misfolded luminal glycoproteins (Oda et al. 2006). Expression of *coiled-coil-helix-coiled-coil-helix domain containing 10* (*CHCHD10*), mitochondrial protein gene in peripheral blood was shown to be pleiotropic with IgG glycosylation. *V-set pre-b cell surrogate light chain 3* (*VPREB3*) gene encodes a protein involved in B lymphocyte maturation (Melchers 2005) and was prioritised for being a member of pathways enriched for IgG glycosylation. Due to the implications for the interaction of SWI/SNF complex with *RUNX1* gene in controlling expression of the genes in haematopoietic cell line (Bakshi et al. 2010), *SMARCB1* together with *VPREB3*, *CHCHD10*, and *DERL3* genes remains a candidate gene for IgG *N*-glycosylation.

The second locus on chromosome 22 harbours three genes including *TGF-beta activated kinase 1* (*MAP3K7*) *binding protein 1* (*TAB1*), *synaptogyrin 1* (*SYNGR1*), and gene encoding the enzyme mannosyl (*β*-1,4-)-glycoprotein *β*-1,4-*N*-acetylglucosaminyltransferase (*MGAT3*) (Klaric et al. 2020). *The SYNGR1* gene encodes an integral membrane protein expressed throughout the nervous system and is likely involved in traffic through the plasma membrane (Stenius et al. 1995; Baumert et al. 1990). Expression of *SYNGR1* (in CD8 cytotoxic T cells and peripheral blood) and *MGAT3* (in CD19 B cells) is pleiotropic with IgG glycosylation (Klaric et al. 2020); however, the most plausible candidate gene in this region is *MGAT3* since the encoded *N*-acetylglucosaminyltransferase III enzyme is known to catalyse the addition of GlcNAc to the core β-mannose residue of *N*-glycans by β1,4-linkage (Taniguchi et al. 2014).

8.4.2 Suggestive Associations

Genome-wide association studies are a powerful tool for detecting genetic associations, but, as all statistical tests, suffer from false-positive associations. Given that in each GWA study millions of association tests are performed (for each variant in the genome), using the classical *p*-value threshold of 0.05 would just by chance result in tens of thousands of associations classified as significant, even when the null hypothesis of no association is true. To mitigate this problem, in a typical GWA study the significance threshold is set to 5×10^{-8}, correcting the "classical" 0.05 threshold for the number of independent tests performed, based on an assumption that there are roughly 1,000,000 independent SNPs in the genome ($0.05/1{,}000{,}000 = 5 \times 10^{-8}$). This does not, however, protect fully from false-positive associations, it merely reduces their number. To further validate the findings from these studies, an additional step is usually performed—repeating the GWAS, on samples collected in cohorts that were not used in the discovery analysis (so-called replication GWAS). Since there is a delicate balance between having a discovery study with as high statistical power as possible, but still leaving some samples aside for replicating the findings, replication GWAS are often performed on several orders of magnitude fewer samples, resulting in a reduced power in replication study. In previous paragraphs, we reported only robust, replicated findings. However, since replication studies are usually underpowered, some of the non-replicated findings might still be true associations. For the interest of readers, we are reporting these findings in the following paragraphs, but stress that these should be considered only for exploratory purposes.

Variants in chromosome 5 region were located in *interleukin 6 signal transducer* (*IL6ST*) (Lauc et al. 2013), a gene which codes for signalling receptor subunit shared by multiple cytokines such as ciliary neurotrophic factor (CNTF), interleukin 6 (IL6), oncostatin M (OSM), and leukaemia inhibitory factor (LIF) (Rose-John 2018). Variants in *IL6ST* were previously linked to rheumatoid arthritis and multiple myeloma susceptibility, as well as components of metabolic syndrome (Stahl et al. 2010; Birmann et al. 2009; Gottardo et al. 2008). However, it must be stressed that this genomic locus was not significant in Klaric et al. (2020). While its biological function might seem relevant for IgG glycosylation, there is no robust evidence for the involvement of *IL6ST* in IgG glycosylation.

The second interval on chromosome 5, spanning *elongation factor for RNA polymerase II* (*ELL2*) gene was one of the newly discovered associations in multivariate GWAS (Shen et al. 2017) and then further confirmed, but not replicated, in the bigger univariate GWAS (Klaric et al. 2020). *ELL2* gene encodes a transcription elongation factor with a role in controlling expression of membrane-associated immunoglobulin by B cells and secretion of immunoglobulin by plasma cells (Santos et al. 2011; Benson et al. 2012). *ELL2* is involved in the regulation of the processing of mRNA transcribed from *IGH* locus, which contains genes coding for heavy chains of immunoglobulins (Martincic et al. 2009). Interestingly, *IGH* locus

was also significantly associated with glycan phenotypes in the same study, indicating the potential biological link between *ELL2* and IgG glycosylation.

First discovered in the multivariate GWAS and rediscovered in the biggest IgG *N*-glycome GWAS, *HLA* (*human leukocyte antigen*) locus on chromosome 6 is an expected association due to the direct role of this gene-dense region in innate and adaptive immunity. It spans more than a hundred genes that have been associated with multiple autoimmune diseases (Shiina et al. 2009). However, due to the complexity of the region, with an extended range of high linkage disequilibrium covering many genes, it is challenging to point to specific variants and genes influencing IgG glycosylation.

A region on chromosome 7 harbours two genes: *diacylglycerol lipase beta* (*DAGLB*) and *KDEL endoplasmic reticulum protein retention receptor 2* (*KDELR2*) (Klaric et al. 2020). *DAGLB* gene codes for a serine hydrolase enzyme involved in proinflammatory signalling in neuroinflammation, with enriched activity in macrophages. *KDELR2* encodes a member of the KDEL receptor family with a role in retrieving ER-resident proteins from the Golgi apparatus back to ER (Capitani and Sallese 2009). Proximity to the strongest glycan-SNP association in the region prioritised the *DAGLB* gene (Klaric et al. 2020) but given the lack of replication of the locus in other cohorts, its role in IgG glycosylation should be considered with caution.

Outer dense fiber of sperm tails 1 (*ODF1*) gene is found in the region on chromosome 8 (Klaric et al. 2020). *ODF1* gene encodes a protein located in outer dense fiber in mammalian sperm tail with a role in spermatogenesis, however, its exact function is not yet fully understood (Amaral et al. 2013). There is no current evidence linking it to the IgG glycosylation process or immune response in general.

A variant on chromosome 11 found in *SUV420H1-CHKA* locus was associated with FA1 glycans in the first IgG *N*-glycome GWAS (Lauc et al. 2013). Histone-Lysine *N*-methyltransferase (*SUV420H1*) gene encodes an enzyme that trimethylates lysine 20 of histone H4, thereby affecting the activity of various genes (Schotta et al. 2004) and, via epigenetic mechanisms, might be involved in proviral silencing in germline and somatic cells (Matsui et al. 2010). *Choline kinase alpha* (*CHKA*) gene is known to be involved in phospholipid biosynthesis and tumour cell growth (Aoyama et al. 2004). Existing evidence of strong associations between glycomics and lipidomics favours *CHKA* as a plausible candidate gene from this region on chromosome 11 since lipid environment is considered to affect the activity of glycosyltransferases (Igl et al. 2011). However, it must be stressed that this association was not confirmed in Klaric et al. (2020) and is therefore not considered robust.

A region on chromosome 16 contains five genes including *component of oligomeric Golgi complex 7* (*COG7*), *Golgi associated*, *gamma adaptin ear containing*, *ARF binding protein 2* (*GGA2*), *glutamyl-tRNA synthetase 2*, *mitochondrial* (*EARS2*), *NADH:ubiquinone oxidoreductase subunit AB1* (*NDUFAB1*), and *ubiquitin family domain containing 1* (*UBDF1*) genes (Klaric et al. 2020). *COG7* gene encodes a member of the Golgi-localised protein complex essential for proper protein *N*-glycosylation. Mutations in *COG7* were indicated in patients with congenital disorders of glycosylation (Wu et al. 2004). On the other hand, *GGA2 g*ene

codes for a member of a protein family with a role in protein trafficking between the *trans*-Golgi network and the lysosome (Hirst et al. 2000). *GGA2* gene was prioritised by pathway enrichment analysis, while the expression of *COG7* in peripheral blood was pleiotropic with IgG glycosylation (Klaric et al. 2020). Given their localisation in the Golgi apparatus, where glycosylation mainly takes place, and prioritisation evidence, both genes remain plausible gene candidates in the region, but have not been replicated in other cohorts.

Mitochondrial genome maintenance exonuclease 1 (*MGME1*) *gene* is found in the locus on chromosome 20 (Klaric et al. 2020). *MGME1* encodes a mitochondrial nuclease which plays an important role in the maintenance and correct metabolism of mitochondrial DNA (El-Hattab et al. 2017). However, besides this gene being the closest to the IgG glycosylation associated SNPs (Klaric et al. 2020), there is no additional evidence for how *the MGME1* gene might affect IgG glycosylation.

Multivariate GWAS approach for IgG glycome resulted in the discovery of a region on chromosome 19 containing the *FUT3-5-6* gene cluster (Shen et al. 2017). The gene cluster codes for fucosyltransferases, enzymes involved in transfer of fucose from donor to acceptor molecules with a role in determining Lewis blood groups (Taniguchi et al. 2014). Furthermore, it was shown that these genes catalyse the addition of antennary fucose (Ma et al. 2006). *Fucosyltransferase 6* (*FUT6*) was previously associated with glycosylation of plasma proteins (Lauc et al. 2010; Sharapov et al. 2019) and was rediscovered in Klaric et al. (2020) as being associated with GP20, a structure that was recently reported to have antennary fucose (Russell et al. 2017), thereby suggesting the potential functional effect of this gene on IgG glycosylation.

Another associated region on chromosome 19 includes *regulatory factor X associated ankyrin-containing protein* (*RFXANK*) gene (Klaric et al. 2020), coding for a subunit of RFX complex, trimeric transcription factor interacting with MHCII promoters and activating the gene expression (Ting and Trowsdale 2002). According to the pleiotropy analysis, the same underlying variant in the locus influences IgG glycosylation and expression of the RFXANK gene in peripheral blood (Klaric et al. 2020). Together with its involvement in regulation of expression of immune system relevant genes, this evidence supports *RFXANK* as a gene candidate for IgG glycosylation, but as all other associations reported in this section, needs to be treated with caution for lack of replication.

8.4.3 Functional Network of Loci Associated with IgG Glycosylation

To understand mechanisms underlying glycosylation of IgG, the natural next step is to ask in which biological pathways are these genes enriched and how the non-glycosylation genes influence glycosylation of IgG.

To put these findings in a biological context, Klaric et al. (2020) applied Data-driven Expression Prioritised Integration for Complex Traits (Pers et al. 2015), a method that tests for enrichment of the list of genes in predefined pathways. The main advantage of this method is that pathways are not only based on known molecular and biochemical pathways, but also integrate information from gene co-expression, mouse gene knock-out studies and protein–protein interaction databases. While there were no statistically significantly enriched pathways below a strict significance threshold, a more relaxed threshold allowing for 20% of false positives uncovered many relevant pathways, with the strongest evidence for enrichment of IgG glycosylation genes in B cell differentiation, activation, and proliferation, abnormal immune system physiology, immunoglobulin production, production of molecular mediators of immune response, thymus hyperplasia, abnormal Peyer's patch morphology (germinal centres responsible for differentiation of B cells in intestine) (Spencer and Sollid 2016), and protein–protein interaction subnetworks of two transcription regulators, serum response factor (SRF) and inhibitor of DNA binding 2 (ID2) (Klaric et al. 2020). This method also provides an insight into tissue enrichment, highlighting that IgG glycosylation-related genes are mostly expressed in haemic and immune system tissues, primarily B-lymphocytes and antibody-producing cells (Klaric et al. 2020).

Genes with the strongest influence on IgG glycosylation were known glycosyltransferase enzymes from the known biological pathway, namely fucosyltransferase FUT8, sialyltransferase ST6GAL1, galactosyltransferase B4GALT1, and MGAT3, an enzyme that produces the bisecting GlcNAc. This finding was not surprising and, in a way, provides a proof of principle that genome-wide association studies can be used to pinpoint genes that regulate IgG glycosylation. The next step was to propose how the other, non-glycosylation genes influence IgG glycosylation through their direct or indirect effect on glycosyltransferases. As we have seen in the previous sections, associated SNPs within glycosyltransferase genes are non-coding and are likely to regulate their expression. With an assumption that a glycosyltransferase and any gene that has an influence on the enzyme (either by controlling its expression, controlling substrate availability, or a different mechanism) will have a similar effect on all glycan traits associated with that enzyme, Klaric et al. (2020) created a functional network of IgG glycosylation and proposed which genes are more likely to influence which glycosyltransferase.

The strongest evidence was found for regulation of expression of *MGAT3* by transcription factors RUNX1 and RUNX3, and chromatin remodelling protein SMARCB1. The network analysis revealed that SNPs from *SMARCB1*, *RUNX1*, *RUNX3* and *MGAT3* loci have strikingly similar effects on IgG glycosylation, suggesting that all of these genes together affect bisecting of IgG *N*-glycans. The strongest associated variant within the *MGAT3* locus is within a binding site of RUNX3 transcription factor. While there was no publicly available information on

RUNX1 binding sites at the time of the study, the two proteins, RUNX1 and RUNX3 are homologous and bind for the same DNA motif. In addition, there was evidence of SMARCB1 and RUNX1 interacting in immortalised T-lymphocytes (Bakshi et al. 2010). Altogether, this suggests that transcription factors RUNX1 and RUNX3, together with the chromatin remodelling protein SMARCB1 could be regulating transcription of *MGAT3*.

The second strongest evidence from the network analysis for non-glycosylation genes influencing expression of glycosyltransferases was found for regulation of expression of *FUT8* by transcription factors IKZF1 and IKZF3. A functional study by Klaric et al. (2020) has shown that knock-down of *IKZF1* in a B cell-derived lymphoblastoid cell line, MATAT6, down-regulates expression of *IKZF3* and upregulates *FUT8* expression, which results in increased IgG fucosylation.

Less evidence and less clear mechanistic insight were found for the remaining two glycosyltransferases, *ST6GAL1* and *B4GALT1*. *HIVEP2* was suggested to regulate expression of galactosyltransferase *B4GALT1* and *ELL2* and *NXPE1-NXPE4 were* suggested to regulate gene expression of sialyltransferase *ST6GAL1*. However, there is no clear mechanistic explanation for either of the two.

8.5 Pleiotropy with Complex Traits and Diseases

IgG glycans are long known to be changed in various diseases and physiological changes, from ageing to autoimmune, inflammatory, and infectious diseases to cancer, being both biomarkers of the disease and biomarkers of its progression [reviewed in Gudelj et al. (2018)]. Indeed, one of the earliest findings related to IgG glycosylation was that of aberrant galactosylation in patients with rheumatoid arthritis (Parekh et al. 1985), followed by extensive research into its effects in ageing (Vanhooren et al. 2007, 2010; Ruhaak et al. 2011; Krištić et al. 2014). While there is increasing evidence that glycans may be involved in every major disease (National Research Council 2012), the mechanisms behind these changes still remain unclear. Genome-wide association studies are also a useful tool in this regard—finding genetic variants that influence both glycosylation and disease risk can help understanding aberrant glycosylation in those diseases. Such variants that influence two or more traits are called pleiotropic. Klaric et al. (2020) investigated pleiotropy of IgG glycosylation-associated variants by exploring which other diseases the same variants were associated with and assessing whether both the disease risk and glycosylation are likely to be regulated by the same underlying causal variant. While there was evidence of shared variants with 83 different diseases, mostly affecting the immune and inflammatory system, but also digestive and neurological system, 3 IgG glycosylation loci and 8 diseases were pleiotropic, suggesting that the same underlying causal variant influences both the disease risk and IgG glycosylation. Namely,

variants associated with IgG agalactosylation within the *IRF1-SLC22A4* were pleiotropic with risk for Crohn's disease, IgG agalactosylation and fucosylation associated variants from the *ORMDL3-GSDMB-IKZF3-ZPBP2* locus were pleiotropic with risk for inflammatory bowel disease, ulcerative colitis, asthma, rheumatoid arthritis, primary biliary cirrhosis and high-density lipoprotein (HDL) cholesterol level, and mono- and digalactosylated core fucosylated traits with bisecting GlcNAc-associated variants from the *CRHR1-SPPL2C-MAPT-ARHGAP27* locus were pleiotropic with Parkinson's disease. For the remaining 75 diseases, it was either impossible to assess pleiotropy for a lack of publicly available summary statistics data or the disease risk was more likely to be controlled by a different underlying causal variant (Klaric et al. 2020). It is important to note that while the two traits can be pleiotropic (regulated by the same underlying causal variant), these types of analyses do not infer the direction of the causality and do not clarify whether the change in glycan levels is causing the disease or the disease is causing the change in glycan levels.

Another point to consider is that, while two traits might be regulated by the same underlying variant, tissue context is also of importance—the same variant can have an effect on a different trait depending on the tissue. For example, the observed pleiotropy of IgG glycosylation and Crohn's disease does not necessarily imply causality—it is possible that the same variant is independently influencing glycosylation of IgG in B cells and Crohn's disease risk in T cells. To further elucidate such paradigms, more detailed analyses of causality, supported by functional follow-up are needed.

8.6 Conclusions

A key insight from genetic studies of IgG *N*-glycome is that genetic regulation of IgG glycosylation is a highly complex process and, apart from the main glycosylation enzymes, involves possibly hundreds of other genes which operate to collectively influence the *N*-glycosylation of IgG. Although each of the variants identified in genetic studies of IgG *N*-glycome makes a small contribution to the IgG glycosylation process, in aggregate they can still explain only a portion of variation in IgG glycans (0.5–21.9%) (Klaric et al. 2020), implicating that further studies, with an even higher number of samples and including variants at the rare allele frequency spectrum are needed. Studying genetic regulation of IgG glycosylation provides not only better insight into this complex process, but through discovering pleiotropic variants, SNPs associated with both glycosylation and complex disease, can also help understanding aberrant glycosylation in those diseases. However, further studies are needed to determine direction of the causality, assessing whether changes in IgG glycosylation are causing the disease or the disease is causing changes in IgG glycosylation.

Compliance with Ethical Standards

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Conflict of Interest AF and OOZ are the employees of Genos Ltd., which specialises in high-throughput glycoanalysis. LK declares that she has no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the studies.

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Chapter 9
Epigenetics of Immunoglobulin G Glycosylation

Marija Klasić and Vlatka Zoldoš

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Abstract Alternative glycosylation of immunoglobulin G (IgG) affects its effector functions during the immune response. IgG glycosylation is altered in many diseases, but also during a healthy life of an individual. Currently, there is limited knowledge of factors that alter IgG glycosylation in the healthy state and factors involved in specific IgG glycosylation patterns associated with pathophysiology. Genetic background plays an important role, but epigenetic mechanisms also contribute to the alteration of IgG glycosylation patterns in healthy life and in disease. It is known that the expression of many glycosyltransferases is regulated by DNA methylation and by microRNA (miRNA) molecules, but the involvement of other epigenetic mechanisms, such as histone modifications, in the regulation of glycosylation-related genes (glycogenes) is still poorly understood. Recent studies have identified several differentially methylated loci associated with IgG glycosylation, but the mechanisms involved in the formation of specific IgG glycosylation patterns remain poorly understood.

Keywords Epigenetic · Gene regulation · DNA methylation · Histone modifications · miRNA · IgG glycosylation

M. Klasić · V. Zoldoš (✉)
Division of Molecular Biology, Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia
e-mail: vzoldos@biol.pmf.hr

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Abbreviations

5-aza-CR	5-azacytidine
5-aza-dC	5-aza-2′-deoxycytidine
ADCC	antibody-dependent cellular cytotoxicity
AFP	α-fetoprotein
CML	chronic myeloid leukemia
CRC	colorectal carcinoma
EWAS	epigenome-wide association study
GlcNAc	*N*-acetylglucosamine
GWAS	genome-wide association study
HCC	hepatocellular carcinoma
IBD	inflammatory bowel disease
IgG	immunoglobulin G
miRNA	microRNA
PBMC	peripheral blood mononuclear cell
TSA	Trichostatin A

9.1 Introduction

Alterations in immunoglobulin G glycosylation are reported in many (patho)-physiological conditions and during aging, but the mechanisms responsible for alternative glycosylation are still poorly understood (Gudelj et al. 2018). The formation of a specific glycosylation pattern is a complex multi-level process involving transcriptional, posttranscriptional, translational, and posttranslational regulation (Neelamegham and Mahal 2016). The importance of the genetic component for IgG glycosylation was discussed in detail in the previous chapter. However, less explored are the epigenetic mechanisms responsible for cell type-specific gene expression profiles and thus specific glycosylation. With the rapid development of sophisticated methods for studying epigenetic mechanisms, especially on the genome-wide level, we are now collecting more and more data with the aim to understand which genes are involved in protein glycosylation, and how they are regulated. Moreover, the combination of glycan data and expression/methylation data of glycogenes in various diseases helps us to understand how these changes might be functionally relevant in pathogenesis (Klasić et al. 2016, 2018; Vojta et al. 2016; Menni et al. 2013; Saldova et al. 2011; Zoldoš et al. 2012; Cummings and Pierce 2014). Nevertheless, our knowledge of how specific IgG glycosylation patterns occur in many diseases and whether these aberrant glycan patterns are a cause or consequence of the disease is not well understood.

9.2 Regulation of Glycosyltransferases by Transcription Factors

Numerous studies have identified transcription factors that control the expression of glycogenes in a cell-specific or tissue-specific manner (Guo and Pierce 2015). For example, the transcription factors E1AF and E2F1 are known to upregulate *B4GALT1*, a gene responsible for galactosylation, in mammalian cells (Zhu et al. 2005; Wei et al. 2010). *ST6GAL1*, the gene encoding a sialyltransferase, is upregulated in liver cells by the transcription factors HNF-1, DBP, Sp-1, and Oct-1 (Svensson et al. 1990; Taniguchi et al. 2000). A recent study by Klarić and colleagues suggests that the transcription factors RUNX1 and RUNX3, together with the chromatin remodeler SMARCB1, regulate the expression of the *MGAT3* gene, which encodes the glycosyltransferase responsible for the addition of bisecting *N*-acetylglucosamine (GlcNAc) to the core of a glycan structure (Klarić et al. 2020). On the other hand, the study by Xu et al. demonstrated that *MGAT3* is downregulated through Wnt/β-catenin signaling in a colon carcinoma cell line (Xu et al. 2011). In the large genome-wide association study (GWAS) of IgG glycosylation, Klarić et al. discovered a possible mechanism involved in core fucosylation of IgG molecules. They performed a knockdown of the transcription factor IKZF1, resulting in an upregulation of the *FUT8* gene and an increase of core fucosylated glycans on IgG secreted by a lymphoblastoid cell line (Klarić et al. 2020). Interestingly, another study showed that the transcription factor HNF1A downregulates *FUT8*, which is responsible for core fucosylation, in liver cells (Lauc et al. 2010). Knock-down of *HNF1A* using RNAi induced a decrease of transcriptional expression in *FUT* genes responsible for antennary fucosylation, and an increase in *FUT8* expression responsible for core fucosylation (Lauc et al. 2010). Overall, these results provide new evidence that the regulation of glycosylation might be tissue-specific and cell-specific. Therefore, further studies are needed to elucidate which transcription factors in B cells are relevant for the establishment of alternative IgG glycosylation during numerous pathological conditions and throughout life.

9.3 Epigenetic Regulation of IgG Glycosylation

In the mammalian genome, DNA methylation occurs predominantly at the fifth carbon of cytosine in CpG dinucleotides. It is established in mammalian early development and maintained during life. Nevertheless, the methylation pattern can change during a life due to environmental factors (Herceg 2007; Martin and Fry 2018). Moreover, DNA methylation patterns are ubiquitously altered in many complex diseases, with changes best characterized in various tumors (Bergman and Cedar 2013). For example, global hypomethylation and promoter

hypermethylation occur in tumors, leading to genomic instability and dysregulation of many genes (Jones and Baylin 2007).

Histone modifications, such as acetylation, methylation, phosphorylation, and ubiquitylation, affect chromatin structure by merely being there (e.g., acetylation adds a negative charge to histones that repels negatively charged DNA, resulting in chromatin relaxation) or by recruiting chromatin remodeling proteins (Bannister and Kouzarides 2011). Numerous studies have shown dysregulation of histone modifications in various diseases, including cancer and autoimmune diseases (Audia and Campbell 2016; Araki and Mimura 2017). Among the affected genes, altered histone modifications have been found in several glycogenes, but regulation of glycogenes by histone modifications is less well understood than regulation by DNA methylation. For example, the expression of *ST6GalNAc6*, a gene encoding the α2→6 sialyltransferase, is downregulated in human colon cancer cells compared to normal epithelial cells. Experiments performed by Miyazaki and colleagues indicated that the *ST6GalNAc6* gene is probably controlled by histone deacetylation in human colon cancer cells, resulting in a decrease in the expression of disialyl Lewis (a) structures (Miyazaki et al. 2004). Another study showed downregulation of the *DTDST* gene in colon cancer cells, but its expression was restored after treatment with histone deacetylase inhibitors, indicating an important role of histone acetylation in the regulation of this gene (Yusa et al. 2010). In addition, several studies showed neural cell-specific regulation of the *Mgat5b* gene by histone acetylation (Kizuka et al. 2011, 2014).

The influence of DNA methylation and histone modifications on protein glycosylation began to be studied intensively about 10 years ago. First studies reported the deregulation of glycogenes via changes in promoter methylation. These first studies used cancer cell lines as a model. Cells were treated with DNA methylation and histone deacetylase inhibitors, such as 5-azacytidine (5-aza-CR), 5-aza-2′-deoxycytidine (5-aza-dC), sodium butyrate, Trichostatin A (TSA), and valproic acid. The treatments resulted in global DNA hypomethylation and global acetylation and consequently changes in glycosylation of membrane and secreted glycoproteins (Klasić et al. 2016; Saldova et al. 2011; Horvat et al. 2012, 2013; Anugraham et al. 2014; Kohler et al. 2016). Treatment of cells with these inhibitors induced deregulation of many glycogenes through direct DNA demethylation and histone acetylation of their promoters or indirectly through epigenetic changes of upstream regulatory factors and yet unknown indirect mechanisms (Fig. 9.1). For example, hypomethylation of the *MGAT3* promoter resulted in an increase of gene expression and change of glycan structures with bisecting GlcNAc on membrane glycoproteins in ovarian carcinoma cells (Kohler et al. 2016). On the other hand, *MGAT5* expression was increased after 5-aza-dC treatment probably due to the activation of specific transcription activator, leading to an increased level of branched glycans on secreted glycoproteins in ovarian carcinoma cells (Saldova et al. 2011). It was also shown that the induced changes in *N*-glycosylation of membrane proteins were reversible upon the recovery of the cells in a medium free of DNA methylation and histone deacetylation inhibitors, suggesting that glycan change, due to epigenetic mechanisms, is reversible (Horvat et al. 2013).

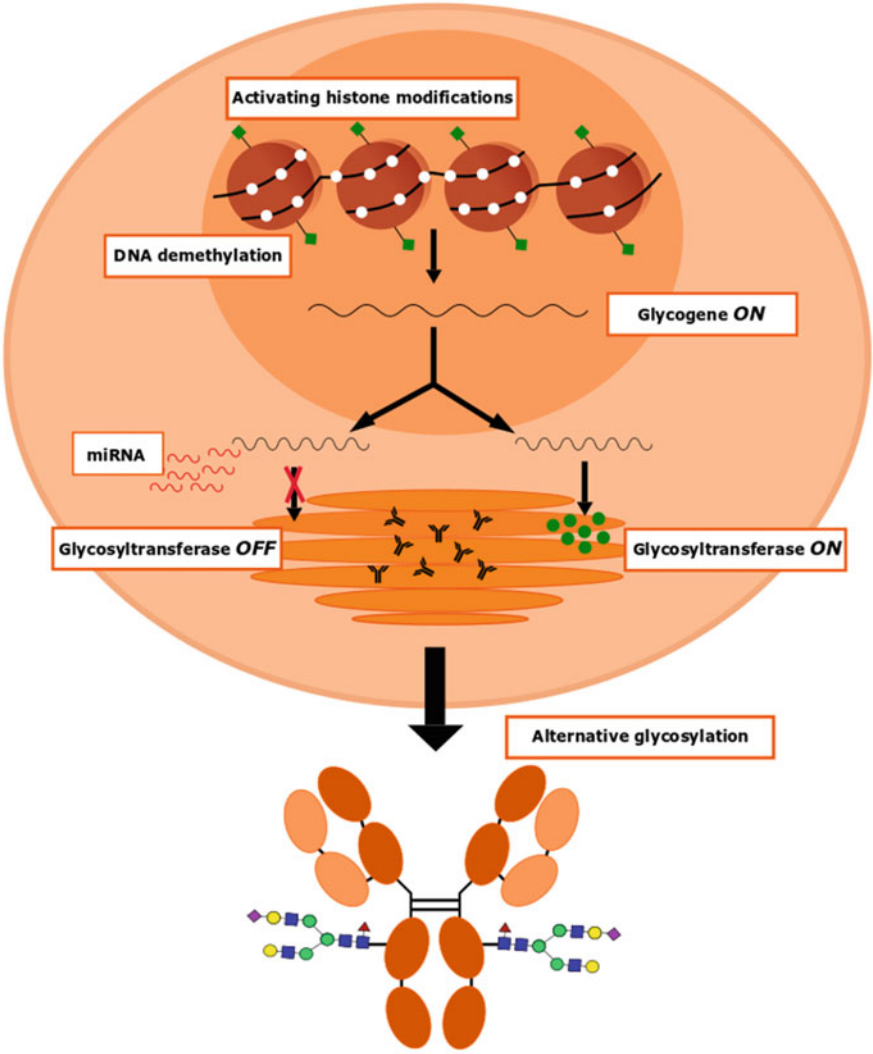

Fig. 9.1 Epigenetic mechanisms involved in alternative glycosylation of immunoglobulin G. Transcriptionally active chromatin leads to the expression of glycogenes. Glycogene mRNA can be processed and translated into a protein or inactivated posttranscriptionally by miRNA molecules, leading to a decrease in protein level. Differences in glycosyltransferase expression can lead to alternative glycosylation of immunoglobulin G. White circles—unmethylated DNA; green rectangles—activating modifications on histones

Epigenetic regulation of the glycogene *MGAT3* has been well characterized in several studies. Treatment of ovarian and hepatocellular carcinoma (HCC) cells with 5-aza-dC resulted in decreased methylation of *MGAT3* promoter and increased level of gene expression. Upregulation of *MGAT3* resulted in altered levels of glycans on

total membrane and secreted glycoproteins. Treatment with 5-aza-dC induced an increase in *N*-glycans with bisecting GlcNAc on the cell membrane glycoproteins of ovarian carcinoma cells, whereas in HCC cells secreted glycoproteins showed less tetraantennary and core fucosylated structures (Klasić et al. 2016; Anugraham et al. 2014; Kohler et al. 2016). To further investigate the effect of DNA methylation on *MGAT3* expression and glycan phenotype, Josipović and colleagues used CRISPR/dCas9-DNMT3A molecular tool to specifically methylate a part of the CpG island of this gene. Targeted methylation led to downregulation of *MGAT3* in an ovarian adenocarcinoma cell line and consequently to a decrease of *N*-glycan structures with bisecting GlcNAc (Josipović et al. 2019).

A large study using publicly available methylation and expression data, combined with wet-lab experiments, revealed ten glycogenes that ubiquitously show aberrant expression patterns in different types of tumors and metastases through CpG methylation (Vojta et al. 2016). For some of them (*GALNT* and *MAN* genes), it was the first report that the change of expression occurs via aberrant promoter methylation. The importance of this study was a discovery of a new group of genes, glycogenes, that contribute to tumors as well as the discovery that aberrant expression of these genes due to aberrant promoter methylation is a way leading to characteristic glycosylation profiles often described in cancer (Stowell et al. 2015). Tumorigenesis and tumor progression are accompanied by significant changes in protein glycosylation. Tumor cells have an altered core of *N*-glycans and branching. For example, glycoproteins on the cell surface may be enriched with fucosylated and hypersialylated glycans during tumor transformation (Miyoshi et al. 2008; Dobie and Skropeta 2021). Changes can also be seen in glycoproteins secreted from cells, e.g., fucosylated form of α-fetoprotein (AFP) is present in the serum of patients with HCC and may serve as a biomarker that distinguishes HCC from other benign liver diseases (Moriya et al. 2013).

Recent GWASs showed that several transcription factors are also associated with changes in glycosylation. The transcription factor HNF1A, which is associated with fucose metabolism and recognized as a master regulator of plasma protein fucosylation, has been shown to be regulated by DNA methylation (Zoldoš et al. 2012). When regulatory CpG sites in the *HNF1A* regulatory region were manipulated using the TET1-CRISPR/dCas9 molecular tool, a decrease of complex glycan structures with core-fucose was reported in the ovarian adenocarcinoma cell line (Josipović et al. 2019).

Data on the influence of epigenetic mechanisms, such as DNA methylation, on the glycosylation of specific proteins, such as IgG antibody, is still very scarce. Glycans on immunoglobulin G have been shown to be stable in individuals during homeostasis but are displaying a great variability in a population (Pučić et al. 2011). Within an individual, IgG glycosylation pattern changes with lifestyle or aging, as well as in a particular disease (Krištić et al. 2014; Yu et al. 2016; Novokmet et al. 2014). Abundant changes in IgG glycosylation have been observed in many autoimmune diseases as well as in tumors (Gudelj et al. 2018; Klasić et al. 2018; Lauc et al. 2013, 2016). It has been shown that the level of agalactosylated glycans decreases in childhood and adolescence, and then increases with age (Parekh et al.

1988). In many autoimmune diseases (e.g., rheumatoid arthritis, inflammatory bowel disease (IBD), and systemic lupus erythematosus), there was a decrease in galactosylated and sialylated IgG glycans and an increase in fucosylated glycans and structures with bisecting GlcNAc. IgG glycosylation is altered in many tumors such as colorectal and hepatocellular carcinoma, lung cancer, multiple myeloma, and ovarian cancer [for a detailed review see Gudelj et al. (2018)]. Indeed, alternative IgG glycosylation is important for the effector function of this antibody and thus for an adequate immune response (Schwab and Nimmerjahn 2013). Therefore, it is extremely important to unravel the layers of regulation of IgG glycosylation and elucidate mechanisms beyond specific changes in disease. Many genes have been linked to IgG glycosylation through GWA studies. In addition to genes encoding glycosyltransferases and glycosydases, many transcription factors, receptors, signaling molecules, and chromatin remodelers have also been associated with IgG glycosylation (Klarić et al. 2020; Lauc et al. 2013; Shen et al. 2017; Benedetti et al. 2017; Wahl et al. 2018a). Epigenetic regulation of IgG glycosylation has just started to be elucidated. The first epigenome-wide association study (EWAS) addressing associations between whole-genome DNA methylation and IgG glycans was performed on the cohort of monozygotic and dizygotic twin pairs. The study identified differentially methylated positions in the *ANKRD11* and *SFRS10* loci associated with glycans with low heritability, and differentially methylated positions in the *NRN1L* and *QPCT* loci associated with IgG glycans with high heritability. These genes have not been previously reported to play a role in IgG glycosylation, but the results suggest that the IgG glycosylation pattern with low heritability may be epigenetically mediated (Menni et al. 2013). The second EWAS, performed on four large cohorts, discovered an association between IgG glycosylation and smoking through changes in the methylation of the *AHRR* gene. That gives rise to environmental influence on IgG glycosylation mediated by DNA methylation (Wahl et al. 2018b). Moreover, Krištić and colleagues found several significant associations between differentially methylated CpG site located 0.8–0.9 kb upstream of the *BACH2* transcription start site and IgG glycans (Krištić et al. 2014). *BACH2* methylation and expression were altered during aging (*BACH2* mRNA downregulation was reported in CD4+ T cells, CD8+ T cells, and CD19+ B cells in healthy people) and SNP in *BACH2* was previously associated with IgG galactosylation (Lauc et al. 2013; Hannum et al. 2013; Chi et al. 2019). The results from this study suggest that changes in IgG glycosylation during aging may be caused in part by changes in the *BACH2* gene expression, leading to an increased inflammation with age (Krištić et al. 2014).

To address the correlation between methylation in promoter regions of genes encoding glycosyltransferases and/or transcription factors associated with IgG glycosylation, Klasić and colleagues investigated possible correlations between *BACH2* and *MGAT3* methylation and IgG glycan profiles from patients with IBD. While *MGAT3* is a classical glycogene with a known function in the glycosylation network, *BACH2* encodes a transcription factor involved in B cell differentiation and maturation (Igarashi et al. 2014). However, its role in IgG glycosylation is still unknown. A study performed on several hundred patients with IBD, including Crohn's disease

and ulcerative colitis, showed a decrease in *BACH2* and an increase in *MGAT3* promoter methylation in peripheral blood mononuclear cells (PBMCs) and CD19+ B cells separated from whole blood (Klasić et al. 2018). While *BACH2* methylation showed no significant correlation with glycan structures, the *MGAT3* promoter methylation correlated negatively with galactosylated and sialylated glycans, as well as with glycan structures with bisecting GlcNAc on IgG molecules in the same IBD patients. These results suggest a possible deregulation of *MGAT3* via DNA methylation in IBD patients leading to a specific IgG glycosylation pattern. Furthermore, the results suggest a not straightforward and complex role of transcription factors in the formation of specific IgG glycosylation patterns (Klasić et al. 2018).

9.4 The Role of miRNAs in Protein *N*-Glycosylation

As discussed above, glycogenes are partly regulated by transcription factors and epigenetic mechanisms, but a number of studies suggest that glycogene expression can in some cases only semi-quantitatively predict the corresponding enzyme activity and glycan expression. The relationship between glycogene expression and glycan structures is non-linear in most cases, suggesting another level of regulation of protein glycosylation, i.e., posttranscriptional regulation via miRNAs (Fig. 9.1) (Neelamegham and Mahal 2016; Thu and Mahal 2019). MiRNAs are small non-coding RNA molecules that act as posttranscriptional regulators of protein expression. They play an important role in developmental processes in eukaryotes and are dysregulated in many diseases (Ardekani and Naeini 2010). Recent studies have identified miRNAs as important regulators of glycosyltransferases expression, which are also highly dysregulated in different diseases. Several glycogenes associated with IgG glycosylation are known to be posttranscriptionally regulated by miRNAs. The *FUT8* gene, which encodes core fucosyltransferase, is regulated by four miRNAs in HCC, leading to dysregulation of protein expression and consequent changes in protein core fucosylation. *FUT8* is overexpressed in HCC cells and HCC tissue, leading to an increase in core fucosylated glycan structures on membrane and secreted glycoproteins. Overexpression of four miRNA molecules (miR-122, miR-34a, miR-26a, and miR-455-3p) targeting the *FUT8* transcript in HCC cells led to downregulation of *FUT8* and core fucosylation on secreted glycoproteins and suppression of HCC cells progression (Bernardi et al. 2013; Cheng et al. 2016). A similar effect of another miRNA molecule was observed in colorectal carcinoma (CRC) cells. Overexpression of miR-198 in CRC cells induced downregulation of FUT8 and inhibited cell proliferation, migration and invasion. These results were confirmed in vivo, where expression of miR-198 inhibited the growth and invasion of CRC in nude mice (Wang et al. 2014). Moreover, core fucosylation is important for IgG antibody function in antibody-dependent cellular cytotoxicity (ADCC) (Li et al. 2017; Pereira et al. 2018). Recent studies discovered altered core fucosylation on IgG molecules in patients with some autoimmune and

alloimmune diseases as well as in tumors (Gudelj et al. 2018). Moreover, glycosyltransferases responsible for galactosylation, sialylation, and addition of bisecting *N*-acetylglucosamine (B4GALT1, ST6GAL1, and MGAT3) are also regulated by miRNAs at the posttranscriptional level in chronic myeloid leukemia (CML) and HCC cell lines (Liu et al. 2016; Han et al. 2018; Huang et al. 2018). While miRNAs have been shown to be important for the differentiation of B cells into IgG-producing plasma cells, current knowledge about miRNA regulation of IgG glycosylation patterns and the influence on its alternative glycosylation in different (patho)physiological states is insufficient (Vigorito et al. 2007). There is increasing evidence that miRNAs control the fine-tuning of glycosyltransferase expression, but our knowledge of the miRNA network for the assembly of specific glycan structures is still incomplete.

9.5 Conclusions

Changes in glycosylation of immunoglobulin G occur during the healthy life of an individual due to environmental factors, but also in many diseases due to homeostasis perturbation. However, the exact mechanisms leading to the formation of specific glycan patterns associated with a particular disease are still poorly understood. There is increasing evidence that both genetic and epigenetic components are involved in alternative IgG glycosylation. Moreover, several GWA studies of IgG glycosylation have found that not only glycosyltransferases and glycosidases are involved in IgG glycosylation, but also many transcription factors, receptors, signaling molecules, chromatin remodelers, etc. Therefore, recent studies used RNAi silencing or novel molecular tools such as CRISPR/dCa9 for activation and/or repression of gene transcriptional activity to elucidate the functional role of these GWAS hits in the regulation of IgG glycosylation. In addition, many studies have shown an epigenetic influence on alternative protein and IgG glycosylation observed in various diseases such as diabetes, chronic inflammatory diseases, and cancer. Many glycogenes as well as some genes encoding transcription factors are regulated by promoter methylation and epigenetic modifications have been shown to influence *N*-glycosylation. While histone modifications have been little studied in terms of protein glycosylation regulation, miRNA molecules appeared to fine-tune glycosyltransferase expression, and as a result, alter protein glycosylation. Several glycogenes associated with IgG glycosylation were shown to be posttranscriptionally regulated by miRNAs. Future challenges lie in uncovering a complex gene network involved in alternative IgG glycosylation ubiquitously involved in pathophysiological conditions, in order to understand molecular mechanisms beyond specific glycan patterns associated with a specific disease and to find new potential diagnostic, prognostic, and therapeutic strategies.

Ethics

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Conflict of Interest Marija Klasić declares that she has no conflict of interest. Vlatka Zoldoš declares that she has no conflict of interest.

Ethical Approval This chapter does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 10
Immunoglobulin G Glycosylation Changes in Aging and Other Inflammatory Conditions

Fabio Dall'Olio and Nadia Malagolini

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Abstract Among the multiple roles played by protein glycosylation, the fine regulation of biological interactions is one of the most important. The asparagine 297 (Asn_{297}) of IgG heavy chains is decorated by a diantennary glycan bearing a number of galactose and sialic acid residues on the branches ranging from 0 to 2. In addition, the structure can present core-linked fucose and/or a bisecting GlcNAc. In many inflammatory and autoimmune conditions, as well as in metabolic, cardiovascular, infectious, and neoplastic diseases, the IgG Asn_{297}-linked glycan becomes less

The gene names and enzyme names are according to HUGO nomenclature rules (https://www.genenames.org/)

F. Dall'Olio (✉) · N. Malagolini
Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy
e-mail: fabio.dallolio@unibo.it

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sialylated and less galactosylated, leading to increased expression of glycans terminating with GlcNAc. These conditions alter also the presence of core-fucose and bisecting GlcNAc. Importantly, similar glycomic alterations are observed in aging. The common condition, shared by the above-mentioned pathological conditions and aging, is a low-grade, chronic, asymptomatic inflammatory state which, in the case of aging, is known as inflammaging. Glycomic alterations associated with inflammatory diseases often precede disease onset and follow remission. The aberrantly glycosylated IgG glycans associated with inflammation and aging can sustain inflammation through different mechanisms, fueling a vicious loop. These include complement activation, Fcγ receptor binding, binding to lectin receptors on antigen-presenting cells, and autoantibody reactivity. The complex molecular bases of the glycomic changes associated with inflammation and aging are still poorly understood.

Keywords Glycosylation in aging · Inflammaging · Intravenous immunoglobulin · Hypogalactosylated antibodies · Glycosyltransferases

Abbreviations

ACPA	Anti-citrullinated protein antibodies
ADCC	Antibody-dependent cell cytotoxicity
AGEs	Advanced glycation end-products
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Antigen-presenting cells
DAMPS	Danger-associated molecular patterns
DC-SIGN	Dendritic cell-specific ICAM-grabbing non-integrin
FcγR	Fcγ receptor
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GWAS	Genome-wide association studies
IVIG	Intravenous immunoglobulin
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
Man	Mannose
MBL	Mannose-binding lectin
NK	Natural killer cells
PAMPS	Pathogen-associated molecular patterns
RA	Rheumatoid arthritis
RF	Rheumatoid factors
SASP	Senescence-associated secretory phenotype

SNP	Single nucleotide polymorphysms
STAT6	Signal transducer and activator of transcription 6
TNF	Tumor necrosis factor

10.1 Premise

The title of this chapter refers to aging as one of the many conditions associated with inflammation. The aging-associated inflammation is referred to as inflammaging and is low-grade, chronic, and asymptomatic. A similar state of low-grade chronic inflammation is common to various pathological conditions, including metabolic and cardiovascular diseases and cancer.

10.2 IgG Glycosylation

The asparagine residue 297 (Asn_{297}) of the IgG heavy chains bears a diantennary complex type glycan whose basic structure can undergo some functionally important variations under physiological and pathological conditions. On the simplest structure depicted in Fig. 10.1 (boxed), an α1,6-linked Fuc residue can be added to the innermost GlcNAc residue. This modification, which is known as "core fucosylation" and is mediated by fucosyltransferase 8 (FUT8), can take place also on other more complex structures, as shown. Another modification that can take place on the simplest structure, as well as on more complex glycans, is the addition of a β1,4-linked GlcNAc residue to the "central" mannose residue. The enzyme responsible for this modification, which is known as "bisecting" GlcNAc, is β1,4 N-acetylglucosaminyltransferase 3 (MGAT3). The two branches of the glycans can be elongated by the addition of one or two β1,4-linked galactose residues and of one or two α2,6-linked sialic acid residues. The addition of galactose can be mediated by a family of β1,4-galactosyltransferases (B4GALTs), although B4GALT3 has been suggested to play a major role (Schwedler et al. 2018). On the other hand, the addition of α2,6-linked sialic acid is mediated by sialyltransferase ST6GAL1 (Dall'Olio 2000). Glycosylation is a stochastic process regulated mainly by the relative abundance of specific glycosyltransferases. This leads to the phenomenon known as microheterogeneity, which means that the sugar structures attached to a specific glycosylation site in a given glycoprotein display a certain degree of variability. For example, the core fucose, the bisecting GlcNAc, and the other modifications mentioned above can be present on some but not all molecules. It should be kept in mind that while the vast majority of plasma glycoproteins are originated by the liver, antibodies are generated and glycosylated by plasma cells. The position to which the two N-glycans are attached to the two heavy chains of IgG allows them to regulate the interaction with Fcγ receptors (FcγR) (Krapp et al. 2003; Barb and Prestegard 2011; Yamaguchi et al. 1760) and their effector functions (Raju

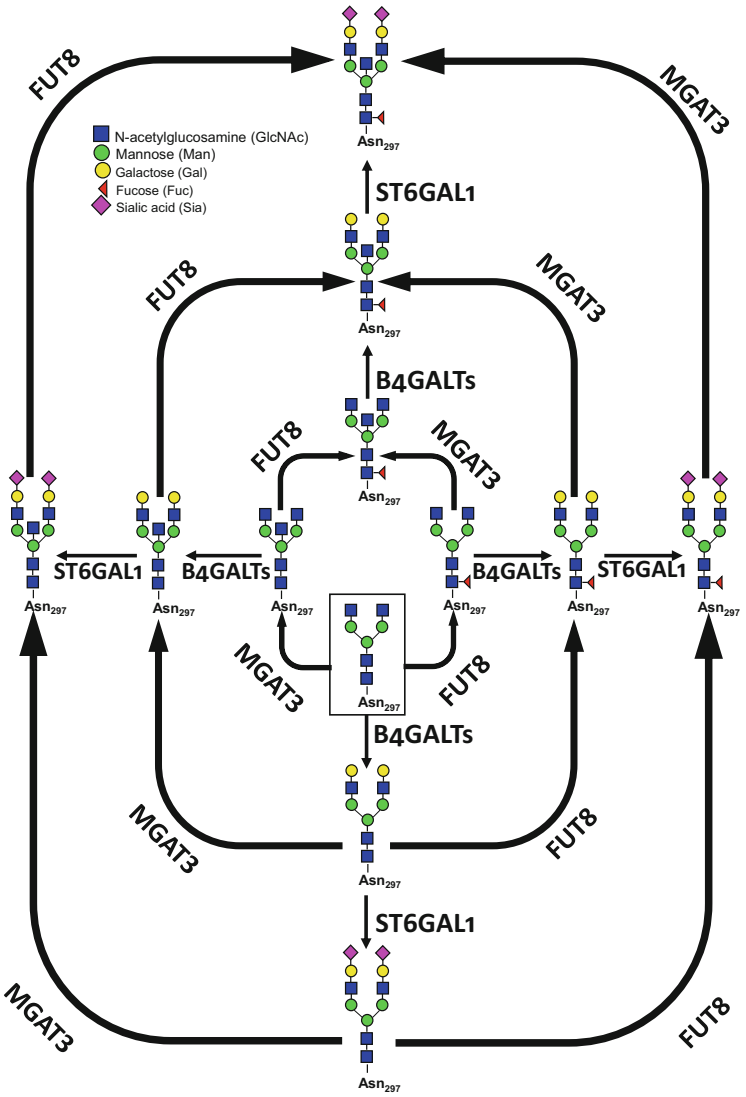

Fig. 10.1 Schematic representation of the different glycoforms which can be attached to Asn_{297} of the IgG heavy chains and their biosynthesis. The simplest structure (boxed) can be modified by the addition of "core fucose" by FUT8 or by addition of "bisecting GlcNAc," mediated by MGAT3. Otherwise, this structure can be β1,4-galactosylated by B4GALTs and successively α2,6-sialylated by ST6GAL1. Addition of core fucose and/or bisecting GlcNAc can take place also on more complex structures. Likewise, galactosylation and sialylation can take place also on core-fucosylated and/or bisected structures. The number of galactose and/or sialic acid residues on these structures ranges from 0 to 2. Owing to the phenomenon of microheterogeneity, all these structures can theoretically be linked to Asn_{297}. Their relative abundance is affected by multiple factors determined by physiological and pathological conditions, as well as the genetic background. Not all the enzymatic reactions can necessarily take place in any order, owing to the substrate specificity of the glycosyltransferase involved

2008). It is well known that glycosylation is a process associated with intracellular membranes. However, it has been shown that it can take place extracellularly, where plasmatic glycosyltransferases are able to glycosylate soluble glycoproteins utilizing sugar-nucleotides contained in platelets and exosomes as donor substrates (Wandall et al. 2012; Jones et al. 2012). In particular, antibodies can undergo extracellular sialylation, catalyzed by plasmatic sialyltransferase ST6GAL1 (Jones et al. 2012, 2016), which utilizes the CMP-sialic acid contained inside platelets as donor substrate (Lee et al. 2014). However, it is not clear which is the real relevance of extracellular glycosylation in shaping the glycosylation pattern of IgG. In fact, the level of α2,6 sialylation of IgG does not correlate with the level of plasmatic ST6GAL1 of healthy subjects (Catera et al. 2016). Moreover, extracellular sialylation plays a very limited role in the sialylation of the bulk of circulating IgG in resting mice (Schaffert et al. 2019) but becomes relevant as a consequence of inflammatory stimuli (Manhardt et al. 2017).

10.3 Changes in IgG Asn_{297} Glycans Associated with Inflammatory Diseases

More than 30 years ago, it was reported for the first time that in the autoimmune inflammatory conditions known as rheumatoid arthritis (RA) and in osteoarthritis Asn_{297}-linked glycans displayed a shift from fully galactosylated structures to GlcNAc-terminating forms (Parekh et al. 1985). Although other modifications of Asn_{297} glycans, such as increased bisecting GlcNAc and core fucosylation, were found to be associated with inflammatory conditions, the hypogalactosylation of the branches is certainly the most relevant (reviewed in (Yamaguchi and Barb 2020)).

Successively, the presence of agalactosylated N-linked chains was found to be associated with different diseases sharing an inflammatory condition (Table 10.1). Besides classical autoimmune/inflammatory conditions, hypogalactosylation of Asn_{297} IgG was found to be associated also with metabolic, cardiovascular and nervous system diseases, as well as with infectious diseases and several neoplasms. The observed changes are not identical among diseases and in some cases allow to distinguish the specific diseases (Axford et al. 2003; Pilkington et al. 1995a; Martin et al. 2001; Liu et al. 2019a, 2020a; Lemmers et al. 2017). In sera of RA patients, reduced galactosylation is detectable also in the N-linked chains of glycoproteins other than IgG (Nakagawa et al. 2007), although hypogalactosylation does not affect glycans attached to IgG light chains (Holland et al. 2006; Mimura et al. 2007; Youings et al. 1996; Bondt et al. 2016) nor to IgA (Field et al. 1994). These findings are not easy to reconcile. In fact, on the one hand, they suggest that inflammatory diseases alter glycosylation in both liver and lymphocytes. On the other hand, they suggest that altered glycosylation is restricted to a specific glycosylation site of IgG produced by B lymphocytes/plasma cells. Decreased galactosylation levels were also associated with mouse models of arthritis (Rook et al. 1991a; Yagev et al. 1993; Kuroda et al. 2001; Bodman et al. 1994; Endo et al. 1993).

Table 10.1 Human diseases associated with plasma hypogalactosylated IgG

Autoimmune/inflammatory diseases	References
Rheumatoid arthritis	Parekh et al. (1985), Axford et al. (2003), Pilkington et al. (1995a), Martin et al. (2001), Su et al. (2020)
Primary osteoarthritis	Parekh et al. (1985), Bond et al. (1997)
Juvenile onset rheumatoid arthritis	Bond et al. (1997), Parekh et al. (1988a), Sumar et al. (1991)
Juvenile idiopathic arthritis	Cheng et al. (2017)
Juvenile chronic arthritis	Flogel et al. (1998)
Psoriatic arthritis	Martin et al. (2001)
Anchylosing spondylitis	Martin et al. (2001), Liu et al. (2019b), Chou et al. (2010)
Spondyloarthropathy	Liu et al. (2020a), Leirisalo-Repo et al. (1999)
Erythema nodosum leprosum	Filley et al. (1989)
Takayasu's arteritis	Hernandez-Pando et al. (1994)
Sjogren's syndrome	Bond et al. (1997)
Systemic vasculitides associated with ANCA	Holland et al. (2002, 2006)
Systemic lupus erythematosus	Pilkington et al. (1995a, 1996a), Vuckovic et al. (2015)
Autoimmune hemolytic anemia	Sonneveld et al. (2017)
Systemic sclerosis	Liu et al. (2020b)
Lambert–Eaton myasthenic syndrome	Selman et al. (2011)
Myositis	Perdivara et al. (2011)
Myastenia gravis	Pilkington et al. (1995a)
Granulomatosis with polyangitis	Kemna et al. (2017)
Guillain–Barre syndrome	Fokkink et al. (2014)
Chronic inflammatory demyelinating polyneuropathy	Wong et al. (2016)
Crohn disease and ulcerative colitis	Bond et al. (1997), Dube et al. (1990), Shinzaki et al. (2008, 2013), Simurina et al. (2018), Theodoratou et al. (2014)
Pancreatitis	Chen et al. (2014)
Alzheimer disease	Lundstrom et al. (2014)
Multiple sclerosis	Wuhrer et al. (2015)
Chronic obstructive pulmonary disease (COPD)	Komaromy et al. (2020)
Metabolic and cardiovascular diseases	
Central adiposity	Liu et al. (2019c)
Ischemic heart disease (in RA)	Troelsen et al. (2007)
Dilated cardiomyopathy	Reinke et al. (2019)
Hypertension	Knezevic et al. (2010), Liu et al. (2018a), Gao et al. (2017)
Nonalcoholic fat liver disease (NAFLD)	Zhao et al. (2018)
Nonalcoholic steatohepatitis (NASH)	Blomme et al. (2011)
Obesity	Nikolac et al. (2014)

(continued)

Table 10.1 (continued)

Autoimmune/inflammatory diseases	References
Type 2 diabetes	Liu et al. (2019a), Lemmers et al. (2017)
Galactosemia	Coman et al. (2010)
Infectious diseases	
HBV hepatitis	Ho et al. (2014)
HBV-associated fibrosis	Gui et al. (2010), Ho et al. (2015)
HCV-associated fibrosis	Mehta et al. (2008)
Adult tuberculosis	Parekh et al. (1989), Rademacher et al. (1988), Liu et al. (2020c), Rook et al. (1994)
Childhood tuberculosis	Pilkington et al. (1996b)
Parasitic infections	de Jong et al. (2016)
Cancers	
Gastric	Kanoh et al. (2004a), Xin et al. (1995), Bones et al. (2011), Qin et al. (2019, 2020)
Prostate	Kanoh et al. (2004b)
Lung	Kanoh et al. (2004a, 2006), Chen et al. (2013)
Breast	Pierce et al. (2010)
Ovarian	Saldova et al. (2007)
Pancreatic	Chen et al. (2014), Shih et al. (2019)
Colon	Liu et al. (2019d, 2020d), Zou et al. (2020), Vuckovic et al. (2016)
Cholangiocarcinoma	Chang et al. (2018)
Neuroblastoma	Qin et al. (2018)
Hematological malignancies	de Haan et al. (2018a)

In many circumstances, IgG glycosylation closely follows disease remission or progression. For example, in HBV hepatitis, the level of agalactosylated IgG decreases after entecavir therapy (Ho et al. 2014). Some conditions are known to cause RA disease remission and a concomitant normalization of IgG Asn_{297} galactosylation. Examples include pregnancy (Rook et al. 1991b; Alavi et al. 2000; van de Geijn et al. 2009; Pekelharing et al. 1988; Bondt et al. 2013), fasting (Kjeldsen-Kragh et al. 1996), and treatment with anti-TNF (Van Beneden et al. 2009; Croce et al. 2007; Pasek et al. 2006; Collins et al. 2013) or anti-IL-6 (Mesko et al. 2012) antibodies. Pregnancy reduces the agalactosylation of IgG per se, independently on RA (Reiding et al. 2017; Bondt et al. 2014). Interestingly, the level of agalactosylated IgG is always lower in the fetus than in its mother (Williams et al. 1995; Kibe et al. 1996), suggesting a kind of selective transport. Nevertheless, agalactosylated antibodies are involved in the mother-to-child transmission of inflammatory conditions, such as systemic lupus erythematosus (Pilkington et al. 1996a) and myastenia gravis (Pilkington et al. 1995b).

Anti-neutrophil cytoplasmic antibodies (ANCAs) are a group of autoantibodies, mainly of the IgG type, against antigens in the cytoplasm of neutrophils, which are produced in various autoimmune inflammatory conditions, but in particular in systemic vasculitis. In some cases, the glycosylation of ANCA antibodies differs

from that of total IgG, providing different information on the disease status. For example, in granulomatosis with polyangiitis, low galactosylation and sialyation in total IgG1 but not in anti-ANCA IgG1 predicts disease reactivation (Kemna et al. 2017). Moreover, information on disease status can be provided by glycosylation of antigen-specific ANCAs. For example, Fc glycosylation of total IgG was significantly reduced in patients with active ANCA-associated vasculitis. Clinical remission was associated with complete glycan normalization for proteinase3-ANCA patients but not for myeloperoxidase-ANCA patients (Lardinois et al. 2019).

IgG glycosylation is influenced also by socioeconomic conditions. In fact, galactosylation levels were generally lower in less affluent countries and in less urban communities, probably reflecting increased immune activation and consequent inflammation (de Jong et al. 2016).

10.4 IgG Glycosylation as a Predictor of Disease Onset, Progression, and Therapy Response

10.4.1 Prediction of Disease Onset

In some circumstances, IgG glycosylation changes anticipate the onset of disease. The changes are not limited to galactosylation, but involve core-fucosylation, bisecting GlcNAc, and sialylation. Examples of changes detectable before the onset of the disease are reported in Table 10.2. Noteworthy, some modifications, including core-fucosylation and sialylation could be different in different diseases.

Table 10.2 Asn_{297} IgG glycosylation changes as predictive factors of disease

Disease	Core fucosylation	Bisecting GlcNAc	Galactosylation	Sialylation	References
Rheumatoid arthritis			Lower		Ercan et al. (2010), Gudelj et al. (2018), Rombouts et al. (2015)
Granulomatosis with polyangiitis			Lower	Lower	Kemna et al. (2017)
Autoimmune thyroid diseases	Lower				Martin et al. (2020a, b)
Ischemic stroke		Higher	Lower	Lower	Liu et al. (2018b)
Cardiovascular diseases				Higher	Menni et al. (2018)
Liver disease	Higher	Higher	Lower		Zhao et al. (2018)
Plasma markers of inflammation	Higher		Lower	Lower	Plomp et al. (2017)

10.4.2 Prediction of Progression and Therapy Response

Good examples of therapy response prediction in autoimmune/inflammatory diseases are provided by the association between low galactosylation and poor response to methotrexate therapy in early RA (Lundstrom et al. 2017) and to TNF blockers in ankylosing spondylitis (Liu et al. 2019b).

Among infectious diseases, in HBV hepatitis, the presence of fully galactosylated IgG bearing core fucose and bisecting GlcNAc is associated with an attenuated liver inflammation. The response to treatment of these patients was unfavorable, probably because of the importance of the inflammatory response in antiviral defense (Ho et al. 2019). The crucial role played by core-fucosylation in regulating the progression of some infectious diseases is demonstrated by the following examples. In Dengue virus-infected patients producing afucosylated IgG1, infection frequently progresses toward hemorrhagic fever and shock because of the enhanced affinity for FcγRIIIA of afucosylated IgG (see below) which triggers platelet reduction (Wang and Ravetch 2019; Wang et al. 2017; Lok 2017). Moreover, in *Fut8*(−/−) mice (whose IgG is not core-fucosylated) infected with *Salmonella typhimurium*, the presence of bacteria colonizing the cecum was increased and the production of specific antibodies was decreased, compared with wild type mice (Zahid et al. 2020). A group of meningococcus-infected children develops sepsis. These patients display lower fucosylation and higher bisection of IgG1 than age-matched healthy controls (de Haan et al. 2018b). HIV infection is associated with an increased level of fucosylated glycans, which is associated with lower antibody-dependent cell-mediated cytotoxicity (ADCC) (Vadrevu et al. 2018).

Among cancers, in cholangiocarcinoma, the presence of fucosylated agalactosylated IgG1 induces metastasis and early recurrence. This is due to the activation by these antibodies of tumor-associated macrophages M2, which exert a tumor-promoting activity (Chang et al. 2018). After bone marrow transplantation, leukemia patients display a low level of IgG galactosylation, which is more similar to their pretransplantation profiles than to profiles of the donors (de Haan et al. 2018a). This suggests that hypogalactosylation is not directly linked to the presence of cancer.

10.5 IgG Glycosylation in Aging

Few years after the discovery of undergalactosylated IgG in serum of RA patients, a similar alteration in IgG of aging people was reported (Parekh et al. 1988b). Successively the hypogalactosylation of IgG in aging was confirmed by Yamada et al. (Yamada et al. 1997) and by many other investigations (reviewed in (Cobb 2020; de Haan et al. 2020)). Besides hypogalactosylation, also bisecting GlcNAc, shows age dependence (Yamada et al. 1997). A gender dependence of these modifications was suggested by a study showing the increase of agalactosylated

structures only in females, while the increase of bisecting GlcNAc was confirmed in both genders (Shikata et al. 1998). Interestingly, the most prominent drop in the levels of galactosylated and sialylated glycoforms in females was observed around the age of 45–60 years when females usually enter menopause (Bakovic et al. 2013; Ercan et al. 2017). In the past years, different high-throughput techniques have allowed the detailed analysis of a very large number of plasma or serum specimens of various cohorts (Dall'Olio et al. 2013). Analysis of Italian and Belgian cohorts by a DNA sequencer used for the separation of N-glycans, revealed an increase of core-fucosylated, agalactosylated biantennary N-linked chains with or without a bisecting GlcNAc and a concomitant decrease of core-fucosylated di-galactosylated structures in people over 60 (Vanhooren et al. 2007, 2008, 2009). The very complex pattern of glycoforms generated by these techniques required focusing on those provided with the highest biological significance. The GlycoAge test is a good example of an index that combines the predictive potential of key glycan structures. This marker of aging is calculated as the Log of the ratio between a core-fucosylated diantennary N-glycan with two terminal GlcNAc residues and an identical glycan with two terminal galactose residues (Vanhooren et al. 2010). The dependence of the galactosylation pattern by the biological age, rather than by the calendar age, is demonstrated by the observation that the GlycoAge test of people affected by accelerated aging (progeroid) syndromes, such as Werner or Cockaine, was similar to that of centenarians, rather than to that of healthy age-matched controls (Vanhooren et al. 2007, 2010). Reduced galactosylation is associated also with Down syndrome, which presents a feature of accelerated aging (Borelli et al. 2015). Unexpectedly, in children the GlycoAge test was not lower than that of young adults (Catera et al. 2016), indicating that the reduced galactosylation starts with adulthood. Japanese semisupercentanarians (mean age 107 years) display, besides an increase of agalacto- and/or bisecting N-glycans, an increase of multibranched and highly sialylated N-glycans (Miura et al. 2015; Miura and Endo 1860). Analysis by MALDI-TOF-MS of subjects enrolled in the Leiden Longevity Study revealed that age-related glycosylation changes were sex-specific only below the age of 60, with young females having higher galactosylation than males, confirming previous results (Bakovic et al. 2013; Ercan et al. 2017).

Crucial questions on the relationship between IgG glycome and aging regard: (1) the value of glycosylation in prediction of longevity; (2) the relative contribution of genome and environment in shaping the glycome; and (3) the variability of the plasma glycome among healthy individuals and its stability over a short period of times. About the first point, changes of IgG glycome allow to explain up to 58% of variance in chronological age, more than telomere length (Kristic et al. 2014). Prediction of longevity has been attributed to low levels of agalactosylated forms containing a bisecting GlcNAc in people below the age of 60 (Ruhaak et al. 2010, 2011). About the second point, a strong indication about the relative contribution of genome and environment to a given trait can be obtained by the study of monozygotic and dizygotic twins. Through this approach, it has been established that about two-thirds of the plasma glycomic traits are genetically determined, while one-third is mainly controlled by the environment (Menni et al. 2013). Glycosyltransferase

polymorphisms do not play a major role in shaping IgG glycome (Knezevic et al. 2009, 2010). About the third point, in the majority of individuals, the N-glycome displays a "normal" pattern, while it is different in a few outliers (Pucic et al. 2010). Over few days, the glycome of healthy individuals undergoes very little or no changes, while minor changes were observed over a 1-year-long period (Gornik et al. 2009). Although aging is associated with increased IgG agalactosylation, the transition between childhood and adolescence is associated with an opposite trend. In fact, core fucosylation and the level of agalactosylated glycans decreased while digalactosylated glycans increased with age, in both plasma and IgG glycome (Pucic et al. 2012).

Experimental systems confirm the age-associated glycomic changes observed in humans. In fact, decreased IgG galactosylation was observed in all seven mice strain examined between 2 and 8 months of age (Bodman et al. 1994). Moreover, caloric restriction, which in mice is associated with extended lifespan (Weindruch et al. 1988), reverts the increase of agalactosylated N-glycans (Vanhooren et al. 2011).

10.6 The Inflammaging Is a Link Between Aging and Inflammation

As previously suggested (Dall'Olio et al. 2013; Dall'Olio 2018), the age-associated inflammatory status known as inflammaging can be the link to explain the largely overlapping glycomic changes observed in inflammatory conditions and aging. The inflammaging is a chronic, low-grade, asymptomatic inflammatory status, which accompanies aging. This condition is multifactorial and depends on the long life exposure to pro-inflammatory stimuli of microbial, environmental, and endogenous origin (Franceschi 2007; Franceschi et al. 2007, 2018). Some of the stimuli putatively triggering inflammaging include (but are not limited to) the pro-inflammatory cytokines released by senescent cells displaying the "senescence associated secretory phenotype" (SASP) (Rodier and Campisi 2011), the danger-associated molecular patterns (DAMPS), released by necrotic or damaged cells, the pathogen-associated molecular patterns (PAMPS) associated with microorganisms and the advanced glycation end-products (AGEs) released by glycated proteins. Senescent cells are more numerous in elderly people, while DAMPS, PAMPS, and AGEs stimulate various receptors of the innate immune system, resulting in its chronic low-grade activation. Although the notion that the glycomic shift observed in aging is somehow related to inflammaging is highly plausible, observations in humans (Ruhaak et al. 2011) and in animal models (Vanhooren et al. 2011), support the possibility that the age-associated N-glycomic shift is regulated by metabolic pathways, independently of the inflammatory status.

10.7 How Altered IgG Glycosylation Drives Inflammation

Glycosylation at Asn_{297} provides a very good example of the fine-tuning of biological functions by glycans. Carbohydrate structures associated with aging and inflammation can sustain inflammation, triggering a vicious self-sustaining inflammatory loop (Dall'Olio et al. 2013). A seminal demonstration of the pathogenic effect of agalactosylated IgG was the demonstration that galactosidase-treated IgG exhibited increased ability to induce arthritis in healthy mice (Rademacher et al. 1994). The mechanisms which could causally link antibody glycosylation with inflammation include modulation of: (1) complement activation; (2) binding to Fcγ receptors; (3) binding to lectin receptors on antigen-presenting cells; (4) formation of anti-IgG autoantibodies. The effect of differential glycosylation on the different biological functions of IgG is summarized in Table 10.3 and depicted in Fig. 10.2.

10.7.1 Activation of Complement Through the Lectin or the Classical Pathways

It has been reported that the ability of hypogalactosylated IgG to activate complement was higher because of a stronger interaction with mannose-binding lectin (MBL), which is the first component of the lectin pathway of complement (Malhotra et al. 1995; Ezekowitz 1995) [reviewed in: (Rudd et al. 2001; Arnold et al. 2006)]. This notion was challenged by the fact that in RA patients deficient for MBL (Stanworth et al. 1998), as well as in MBL-deficient mice (Nimmerjahn et al. 2007), the pro-inflammatory activity of agalactosylated IgG was not impaired. Moreover, agalactosylated IgG from the plasma of patients affected by inflammatory bowel diseases failed to activate the lectin complement pathway (Nakajima et al. 2011). A study in a mouse model also excludes a role for differential galactosylation in complement activation through the lectin pathway (Ito et al. 2014). Regarding the activation of the classical complement pathway, it has been reported that high galactosylation (Peschke et al. 2017; Dekkers et al. 2017) and sialylation (Zabczynska et al. 2020) of IgG Asn_{297} increased binding and complement activation. This finding was unexpected, considering the pro-inflammatory role of complement, and remains to be clarified (Dekkers et al. 2018a).

10.7.2 Binding to Fcγ Receptors

The most relevant members of the IgG Fc receptors (FcγR) are the following: FcγRI (CD64), a high-affinity receptor expressed mainly on macrophages and neutrophils, very important for phagocytosis; FcγRIIIA (CD16), a low-affinity receptor expressed on NK and macrophages, very important for ADCC; FcγRIIB (CD32),

Table 10.3 Effect of glycosylation modifications on IgG function

		Core fucosylation	Bisecting GlcNAc	Galactosylation	Sialylation
Biological functions	Complement activation via lectin pathway			Down (Malhotra et al. 1995)	
	Complement activation via classical pathway	Unchanged (Niwa et al. 2005)	Unchanged (Dekkers et al. 2017)	Up (Peschke et al. 2017; Dekkers et al. 2017; Kiyoshi et al. 2018; Boyd et al. 1995; Hodoniczky et al. 2005)	Up (Dekkers et al. 2017; Zabczynska et al. 2020) Down (Quast et al. 2015)
	ADCC	Down (Dekkers et al. 2017; Niwa et al. 2005; Shields et al. 2002; Shinkawa et al. 2003; Satoh et al. 2006; Iida et al. 2006, 2009; Bruggeman et al. 2017; Peipp et al. 2008; Dekkers et al. 2018b; Patel et al. 2019; Falconer et al. 2018; Sakae et al. 2017; Harbison and Fadda 2020; Edri-Brami et al. 2012; Wirt et al. 2017; Tsukimura et al. 2017; Zhou et al. 2019)	Up (Umana et al. 1999; Davies et al. 2001; Hodoniczky et al. 2005)	Up (Kumpel et al. 1995; Thomann et al. 2015) Up (associated with hypofucosylation) (Dekkers et al. 2017; Kiyoshi et al. 2018)	Down[a] (Zabczynska et al. 2020; Li et al. 2017; Scallon et al. 2007) Unchanged (Thomann et al. 2015)
	Phagocytosis	Down (Shibata-Koyama et al. 2009)		Up (Kumpel et al. 1995)	
Receptor binding	Inhibitory FCγRIIB (CD32)			Up (Karsten et al. 2012)	Down (Tanigaki et al. 2018)
	High-affinity FcγRI (CD64)	Unchanged (Bruggeman et al. 2017)			Unchanged (Thomann et al. 2015)
	Low-affinity FcγRII (CD32)	Down (Subedi and Barb 2016)		Unchanged (Groenink et al. 1996) Up (Thomann et al. 2015)	Unchanged (Thomann et al. 2015)

(continued)

Table 10.3 (continued)

		Core fucosylation	Bisecting GlcNAc	Galactosylation	Sialylation
	Low-affinity FcγRIII (CD16)	Down (Dekkers et al. 2017; Bruggeman et al. 2017; Wirt et al. 2017; Tsukimura et al. 2017; Subedi and Barb 2016)	Up (Davies et al. 2001) Unchanged (Dekkers et al. 2017)	Up (Adler et al. 1995; Thomann et al. 2015) Unchanged (Peschke et al. 2017; Subedi and Barb 2016)	Up (Adler et al. 1995) Unchanged (Dekkers et al. 2017; Thomann et al. 2015)
	DC-SIGN		Down (Yabe et al. 2010)	Down (Yabe et al. 2010)	Up (Anthony et al. 2008a, 2011)

[a]Only if core-fucose is present

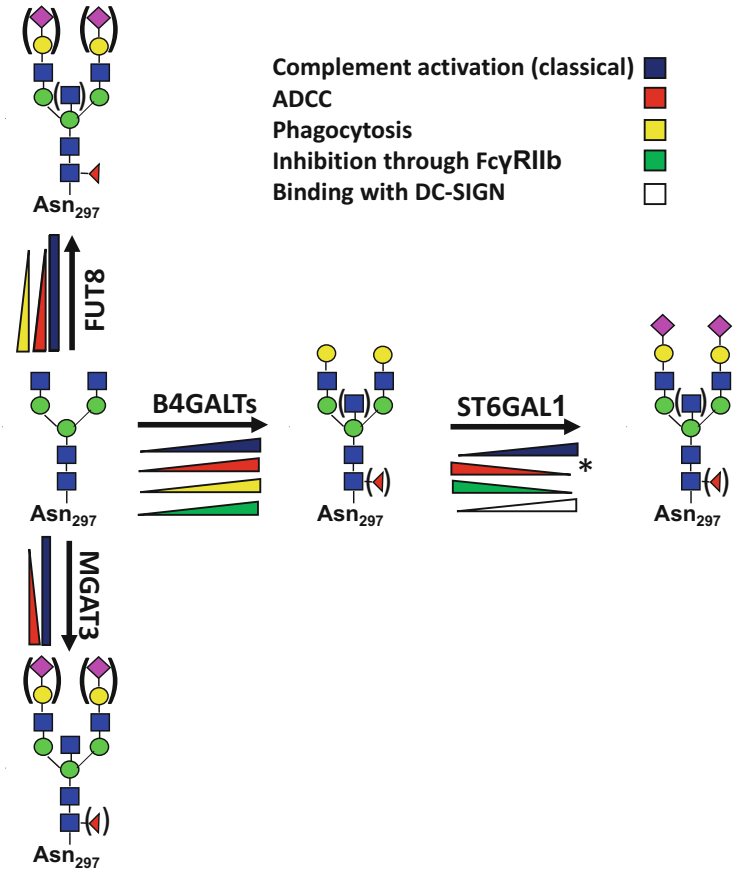

Fig. 10.2 Schematic representation of the functional impact(s) of specific carbohydrate structures on IgG Asn_{297}. The four modifications considered are: core fucosylation, bisecting GlcNAc, galactosylation, and sialylation. Where indicated in parenthesis, the structures can be present or not on that structure. The five IgG functions considered are: complement activation (classical pathway) (blue); ADCC (red); phagocytosis (yellow); immune inhibition through FcγRIIb (green); binding to DC-SIGN (white). Rectangles indicate that this function is not affected by that carbohydrate modification. Triangles indicate that the function is higher in the carbohydrate structure close to the base. *ADCC is reduced by sialylation only if core-fucosylation is present

a low-affinity receptor expressed by B cells, macrophages and dendritic cells which provides feedback inhibitory responses. Glycosylation of IgG at Asn_{297} plays a major role in regulating the binding of IgG to Fcγ receptors of the different classes (Nimmerjahn et al. 2007; Nimmerjahn and Ravetch 2006, 2008; Albert et al. 2008; Li et al. 2017; Karsten et al. 2012). A major role is played by core-fucosylation which inhibits ADCC, mainly mediated by low-affinity FcγRIII on natural killer (NK) cells (Dekkers et al. 2017; Niwa et al. 2005; Shields et al. 2002; Shinkawa et al. 2003; Satoh et al. 2006; Iida et al. 2006, 2009; Bruggeman et al. 2017) (Table 10.3).

However, low fucose enhances only NK-mediated ADCC, while neutrophil-mediated ADCC is enhanced by high fucose (Peipp et al. 2008), probably because it is mediated mainly by a different receptor. Glycosylation of FcγRs modulates the binding of IgG glycoforms (Hayes et al. 2014) because of interactions between the carbohydrate chains of the antibody and of the receptor (Ferrara et al. 2011). In particular, the core fucosylation of Asn_{297} of IgG hinders its interaction with Asn_{162} glycan of FcγRIII, lowering the strength of the IgG- FcγRIII receptor interaction (Dekkers et al. 2018b; Patel et al. 2019; Falconer et al. 2018; Sakae et al. 2017; Harbison and Fadda 2020). The finding that the glycan structures displayed by Asn_{162} of FcγRIII from NK cells of different individuals are highly variable and displaying very different ability to bind IgGs, provides an additional level of variability of immune functions among individuals (Patel et al. 2019).

Regarding the role of bisecting GlcNAc, it has been reported to increase binding of IgG to FcγRIII, enhancing ADCC (Umana et al. 1999; Davies et al. 2001) or to leave it unchanged (Dekkers et al. 2017). Galactosylation has been reported to be necessary for IgG binding to FcγRIII and ADCC (Adler et al. 1995; Kumpel et al. 1995; Kiyoshi et al. 2018), particularly when IgG is hypofucosylated (Dekkers et al. 2017). However, the binding with CD32 does not appear to be modulated by terminal galactose (Groenink et al. 1996). Fully galactosylated IgG that are produced during pregnancy cross the placenta better and provide the fetus and the newborn infant with antibodies optimized for NK ADCC (Pillai 2019). The IgG Fc glycosylation patterns of preterm infants display a more pro-inflammatory phenotype that is not observed in their mothers, suggesting an enrichment process. These IgGs may contribute to the increased risk for sustained inflammatory diseases in preterm infants (Twisselmann et al. 2018). The presence of α2,6-linked sialic acid has been reported to increase (Adler et al. 1995) or to leave unaffected (Dekkers et al. 2017) the binding to FcγRIII. Functionally, higher sialylation decreases ADCC (Zabczynska et al. 2020; Scallon et al. 2007). These apparently inconsistent results could be reconciled considering that sialylation exerts a relevant inhibition of ADCC only when core fucose is present (Li et al. 2017). The level of sialylation of antibodies elicited by vaccination toward influenza virus hemagglutinin positively affects the efficacy of the response because it induces the expansion of B lymphocytes carrying B cell receptors with higher affinity (Wang et al. 2015).

10.7.3 Binding on Lectin Receptors of Antigen-Presenting Cells: Role in the Intravenous Administration of High Doses IgG (IVIG)

Antigen-presenting cells (APC), in particular macrophages and dendritic cells, express on their surface various lectin receptors able to bind a myriad of carbohydrate ligands. Among the most relevant are the mannose-binding receptor and the C-type lectin DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-

Grabbing Non-integrin). The interaction between hypogalactosylated IgG with the mannose-binding receptor (Dong et al. 1999) and DC-SIGN (Yabe et al. 2010) has been reported to be increased.

An increased binding to DC-SIGN of α2,6-sialylated IgG has been reported as an essential step of the mechanisms underlying the anti-inflammatory effect of high doses IgG (IVIG) (Anthony et al. 2008a, 2011). Several autoimmune diseases can be beneficially, although transiently, treated with the intravenous administration of high doses IgG (IVIG) (Baerenwaldt et al. 2010). This treatment induces a functional silencing program similar to anergy in human B cells (Seite et al. 2014). Several lines of evidence indicate that the anti-inflammatory effect of IgG is attributable only to the minor α2,6-sialylated fraction of IgG (Kaneko et al. 2006; Anthony et al. 2008b), which allows interaction with the mouse lectin SIGN-R1 and its human orthologue DC-SIGN (Anthony et al. 2008a).

A model based on mouse experiments proposed that after binding of α2,6-sialylated antibodies to DC-SIGN, dendritic cells release IL-33 which induces the expansion of IL-4 producing basophils (Schwab et al. 2014a; Fillatreau 2014). The two Th2 cytokines upregulate the inhibitory FcγRIIb receptor in macrophages, resulting in the inhibition of inflammation (Anthony et al. 2011). As previously mentioned, glycosylation has been shown to take place extracellularly in some circumstances and could play a role in these mechanisms. In fact, IgG extracellularly glycosylated by soluble galactosyltransferases and ST6GAL1 are able to mimic the effects of IVIG through DC-SIGN, STAT6 signaling, and FcγRIIb (Pagan et al. 2018). In this process, the sialylation of pathogenic IgG was localized at the site of inflammation, because of the local availability of nucleotide-sugar donors released by platelets (Pagan et al. 2018). Thus, inflammation activates a self-inhibiting mechanism which, through the elevation of plasmatic ST6GAL1 (Lammers and Jamieson 1986) and the local presence of CMP-sialic acid donor leads to the extracellular α2,6-sialylation of Asn_{297}-linked IgG glycans, activating an inhibitory Fcγ receptor; this mechanism is exploited by IVIG. In addition, IVIG protects from experimental allergic bronchopulmonary aspergillosis via a sialylation-dependent mechanism, through reduced recruitment of eosinophils and inhibition of Th2 and Th17 responses, with an enhanced involvement of regulatory T cells and IL-10 (Bozza et al. 2019). Classical anaphylactic reactions are mediated by IgE. However, high allergen levels have the potential to mediate anaphylaxis through the binding of allergen-specific IgG to FcγRs on different immune cell types (Miyajima et al. 1997). This type of anaphylaxis can be inhibited by IgG, through a sialylation-dependent mechanism (Epp et al. 2018). However, this view on the mechanisms underlying IVIG is not unanimously accepted (Novokmet et al. 2014; Schwab et al. 2014b; Ballow 2014) and other mechanisms are probably operating. For example, a Fab-dependent but sialylation-independent mechanism through which IVIG exerts anti-inflammatory effects is based on stimulation of autophagy by dendritic cells and M1 macrophages (Das et al. 2020). The picture cannot be considered complete without mentioning recent papers questioning the proposed mechanism (Nagelkerke and Kuijpers 2014). In particular, it has been reported that IgGs do not interact with DC-SIGN, regardless of their glycosylation state (Temming et al. 2019; Yu et al.

2013), that basophils are not required (Campbell et al. 2014), that the amount of IL-33 released is not sufficient (Sharma et al. 2014) and that IgG sialylation is not necessary for T cells reciprocal modulation (Othy et al. 2014), phagocytosis by macrophages (Nagelkerke et al. 2014) or amelioration of murine immune thrombocytopenia (Leontyev et al. 2012).

An unusual mechanism has been claimed to play a role in cancer immunosuppression. Cancer cells could ectopically produce IgG which are abundantly sialylated. The binding of these antibodies to inhibitory sialic-acid receptors of the Siglec family would promote immunosuppression (Wang et al. 2019).

10.7.4 Anti IgG Autoantibodies

The presence of anti-IgG antibodies characterizes RA and other autoimmune diseases. These antibodies, which are often referred to as rheumatoid factors (RF), are in some cases directed against agalactosylated IgG (Mimura et al. 2004, 2005; Nishijima et al. 2001; Das et al. 2004; Maeno et al. 2004; Matsumoto et al. 2000; Soltys et al. 1994; Imafuku et al. 2003) (reviewed in (Goulabchand et al. 2014)). Some studies proposed that hypogalactosylation would promote the formation of aggregates of IgG and RF (Matsumoto et al. 2000; Leader et al. 1996), although this conclusion was not supported by others (Soltys et al. 1994; Imafuku et al. 2003; Falkenburg et al. 2017). Anti–citrullinated protein antibodies (ACPA) are highly specific autoantibodies for a subgroup of RA patients who have a severe erosive disease. An involvement of hypogalactosylated ACPA in disease pathogenesis is suggested by the following observations. (1) The concentration of hypogactosylated IgG1 ACPA is higher in the synovial fluid than in plasma of early RA patients (Scherer et al. 2010). (2) The level of IgG galactosylation was much lower in ACPA than in the rest of IgG molecules (Ercan et al. 2010). (3) The concentration of ACPA correlates with that of hypogalactosylated antibodies in RA patients (Schwedler et al. 2018). (4) In RA women with ACPA, the pregnancy-induced increase of IgG galactosylation affected ACPA and not total IgG (Bondt et al. 2018).

Sialylation of IgG plays an anti-inflammatory key role even through autoantibodies. This notion is supported by the following observations. In both human RA patients and mouse models of arthritis, the sialylation level of IgG is lower; consistently, sialylation of anti-collagen antibodies attenuates their arthritogenic activity (Ohmi et al. 2016). To explain the higher risk of developing RA by postmenopausal women, it has been proposed that, like in mice, estrogens activate ST6GAL1 in antibody-producing cells. Decrease in estrogens after menopause would lead to decreased α2,6 sialylation of IgG (Engdahl et al. 2018). In mouse models of lupus nephritis and RA, the presence of sialylated IgGs directed against self-antigen reduced inflammation through reduction of inflammatory T helper cells (Bartsch et al. 2018). In RA patients, desialylated, but not sialylated, immune complexes enhance osteoclastogenesis, while the Fc sialylation of IgG autoantibodies regulates

the bone architecture (Harre et al. 2015). The anti-inflammatory role of sialylated IgG is confirmed also in glomerulonephritis (Otani et al. 2012).

10.8 Molecular Bases of N-Glycosylation Changes

A number of investigation report that the glycosylation changes associated with inflammatory diseases and aging are specific for Asn_{297} of IgG, while other studies report changes also in glycans linked to the light chains and in non IgG glycoproteins. Investigations on the enzymatic bases of IgG hypogalactosylation have utilized total lymphocyte populations or isolated B lymphocytes from human inflammatory conditions and mouse experimental systems. The studies summarized below are often conflicting and a conclusive evidence on the molecular bases of glycosylation changes of IgG is still lacking. The primary limit of these studies is in the use of B lymphocytes, rather than plasma cells, as an enzyme source. The first are more abundant and easy to obtain than the latter, but their biosynthetic apparatus is not necessarily representative of that of plasma cells, which are the main source of circulating antibodies. Lymphocytes from RA patients can produce in vitro hypogalactosylated IgG (Bodman et al. 1992), while an inverse relationship between hypogactosylated IgG and the level of B4GALTs has been reported by some studies (Axford et al. 1987, 1992; Alavi and Axford 1995). On the other hand, other studies reported a similar galactosyltransferase level in B lymphocytes of RA patients and healthy controls (Furukawa et al. 1990; Keusch et al. 1998; Delves et al. 1990; Jeddi et al. 1996). The importance of environmental factors in the regulation of IgG galactosylation is demonstrated by the fact that all-trans retinoic acid is able to modify specifically galactosylation of IgG1, without affecting glycosylation of other glycoproteins (Wang et al. 2011). Conflicting results have been obtained also from murine experimental models. In splenic lymphocytes of the arthritis-prone MLR lpr/lpr mice, the level of B4GALT1 transcript was found to be reduced (Jeddi et al. 1994, 1996). However, a lower galactosyltransferase activity was detected in peripheral, but not in splenic B lymphocytes in mouse models of arthritis (Axford et al. 1994; Alavi et al. 1998). B4GALT activity was found to be lower in hybridomas producing rheumatoid factors than in hybridomas secreting irrelevant antibodies (Axford et al. 1994).

As previously mentioned, the production of α2,6-sialylated IgG is a part of an anti-inflammatory response. In mice, these anti-inflammatory α2,6-sialylated IgG can be produced against T cell-independent antigens or against T cell-dependent antigens in a tolerogenic microenvironment through modulation of ST6GAL1 expression in plasma cells (Hess et al. 2013; Oefner et al. 2012). The increased level of bisecting GlcNAc associated with aging could be at least in part due to the fact that agalactosylated biantennary N-linked chains, which accumulate in aging, are a preferred substrate for MGAT3 (Narasimhan 1982), which is the only enzyme mediating the addition of bisecting GlcNAc (Ihara et al. 1993). On the other hand,

promoter methylation of *MGAT3* gene correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease (Klasic et al. 2018).

Besides mechanistic studies, the bases of IgG glycome changes have been investigated through genomic and glycomic approaches. Genome-wide association studies (GWAS) indicated a link between given glycosidic traits and genetic loci associated with glycosyltransferases and with autoimmune and inflammatory diseases (Lauc et al. 2013). GWAS identified an association of agalactosylated sugar structures and single nucleotide polymorphisms (SNPs) in the loci *FUT8* and *ESR2*, which encodes estrogen receptor β, but not in *MGAT3* or *B4GALT1* (Lauc et al. 2009). Although the association between polymorphisms in glycosyltransferase genes and specific glycomic traits is rarely strong, polymorphisms in several transcription factors controlling key glycosyltransferases and inflammatory diseases have been identified. For example, *FUT8* has been shown to be associated with polymorphism in the *HNF1A* gene, encoding the transcription factor HNF1α (Sharapov et al. 2019; Lauc et al. 2010), and *IKZF1* (Klaric et al. 2020), while *MGAT3* is under the control of transcription factors RUNX1 and RUNX3 (Wahl et al. 2018).

10.9 Conclusions

After more than three decades of intensive investigation in the field, it is clear that a few glycomic traits of IgG are associated with inflammatory/autoimmune, metabolic, and infectious diseases, aging and cancer. Frequently, these changes precede disease onset and revert upon disease remission. The conditions associated with these IgG glycosylation changes share an inflammatory status which, in the case of aging, is referred to as "Inflammaging." Through different mechanisms, these aberrantly glycosylated IgG can fuel inflammation, triggering a vicious loop. Thus, it is very likely that the Asn_{297} glycosylation changes are intimately associated with the very complex mechanisms governing inflammation, even though the detailed molecular links between control of glycosylation in B lymphocytes and plasma cells and inflammation remain elusive. Considering the causal role of these glycans in sustaining or inhibiting inflammation, the full comprehension of their biosynthesis will provide a new tool for the manipulation of the inflammatory process.

Compliance with Ethical Standards

Conflict of Interest Author F.D declares that he has no conflict of interest. Author N.M. declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 11
Estrogen-Driven Changes in Immunoglobulin G Fc Glycosylation

Kaitlyn A. Lagattuta and Peter A. Nigrovic

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Abstract Glycosylation within the immunoglobulin G (IgG) Fc region modulates its ability to engage complement and Fc receptors, affording the opportunity to fine-tune effector functions. Mechanisms regulating IgG Fc glycans remain poorly understood. Changes accompanying menarche, menopause, and pregnancy have long implicated hormonal factors. Intervention studies now confirm that estrogens enhance IgG Fc galactosylation, in females and also in males, defining the first pathway modulating Fc glycans and thereby a new link between sex and immunity. This mechanism may participate in fetal-maternal immunity, antibody-mediated inflammation, and other aspects of age- and sex-specific immune function. Here

K. A. Lagattuta
Harvard-MIT MD-PhD Program, Harvard Medical School, Boston, MA, USA

P. A. Nigrovic (✉)
Division of Immunology, Boston Children's Hospital, Boston, MA, USA

Division of Rheumatology, Inflammation, and Immunity, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
e-mail: peter.nigrovic@childrens.harvard.edu

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we review the changes affecting the IgG Fc glycome from childhood through old age, the evidence establishing a role for estrogens, and research directions to uncover associated mechanisms that may inform therapeutic intervention.

Keywords Estrogen · Antibody · Glycan · Pregnancy · Autoimmunity · Sex

Abbreviations

ADCC	Antibody-dependent cellular toxicity
β4Gal-T1	β-1,4-galactosyltransferase 1
BCR	B cell receptor
DNA	Deoxyribonucleic acid
E1	Estrone
E2	17(beta)-estradiol
E3	Estriol
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogen response element
ERRγ	Transcription factor estrogen-related receptor gamma
Fab region	Antigen-binding fragment
Fc region	Crystallizable fragment
FcRn	Neonatal Fc receptor
G0	Agalactosylated
G0F	Agalactosylated, fucosylated, non-bisected
G0FB	Agalactosylated, fucosylated, bisected
G1	Monogalactosylated
G2	Digalactosylated
GALE	UDP-galactose 4′-epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridyltransferase
GlcNAc	*N*-acetyl-glucosamine
GnRH	Gonadotropin-releasing hormone
GWAS	Genome-wide association study
HT-SELEX	High-throughput systematic evolution of ligands by exponential enrichment
Ig	Immunoglobulin
IL-6	Interleukin 6
IL-21	Interleukin 21
JIA	Juvenile idiopathic arthritis
MANOVA	Multivariate analysis of variance
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
N-glycan	Oligosaccharide attached to the nitrogen atom of an asparagine residue

NK cells	Natural killer cells
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
St6Gal-T1	ST6 β-galactoside α-2,6-sialyltransferase 1
UDP	Uridine diphosphate
UMP^{2-}	Uridine monophosphate
UPLC	Ultra-performance liquid chromatography

11.1 Introduction

The "omics" revolution has unveiled the staggering diversity and specificity of the immune system at an ever-higher resolution. The breadth of receptor diversity achieved by stochastic DNA rearrangement and somatic hypermutation pertains to antigen recognition, helping to define which immune cells and molecular programs are called to respond to an environmental challenge. However, the immune system must also carefully regulate the downstream pathways engaged by antigen-specific mechanisms to ensure that the immune response remains appropriate to the nature and extent of the threat. Balancing the risks of infection, autoimmunity, and autoinflammation, the immune system responds with the "right cells," and "right severity," at the "right place" and "right time"—an intersection we might call *situationally appropriate reactivity*.

It is reasonable to suspect that what is "right" might change with context and over the course of a lifetime. One moment of the unique challenge is pregnancy, during which a female must maintain her own immune surveillance while simultaneously tolerating and also defending an evolving foreign body within her. Here, situationally appropriate reactivity likely requires a recalibration of immune mechanisms.

One well-known immunomodulating factor is the antibody Fc region, serving to direct humoral responses to specific cell types, effector mechanisms, and compartments. Distinct Fc heavy chains define antibody classes: at a first approximation, IgM for early responses and complement activation, IgA for mucosal immunity, IgE for mast cell immunity, and IgG for most other functions, specified further through subclasses IgG1-IgG4. The characteristics of each of these distinct protein backbones are further refined by post-translational variation: glycosylation within the Fc region.

Position 297 of each IgG heavy chain holds an asparagine (N) residue decorated with a biantennary ("2-armed") glycan varying in length from 7 to 13 sugar residues. The core of this N-glycan is comprised of 4*N*-acetyl-glucosamines (GlcNAc) and 3 mannose molecules. The GlcNAc closest to the core is usually but not invariably fucosylated, and an additional GlcNAc bisects the two glycan arms in ~10% of IgG Fc glycans. The core GlcNAc terminating each of the two arms can receive a galactose, defining glycan families termed G0, G1, or G2 (for a-, mono-, and di-

galactosylation, respectively). Each galactose can in turn be extended by sialylation, giving rise to glycans bearing zero, one, or two sialic acids. Together with minor species, there are over 60 different glycoforms an IgG Fc glycan could assume, approximately 30 of which occur with appreciable frequency.

Glycoform variation in the Fc region, as compared to the Fab region, is of particular interest because it impacts core antibody effector functions. Contained within the "cage" formed by the two heavy chains, glycans maintain the quaternary structure required for interaction with complement and Fc receptors (Sondermann et al. 2000; Barb and Prestegard 2011; Subedi and Barb 2015). Without Fc glycans, IgG cannot interact with complement or most Fc receptors (Nose and Wigzell 1983). However, defining structure-function correlations for individual IgG Fc glycans has proven complex. The four IgG subclasses differ in heavy chain sequence, and lessons learned with one form may not apply to another. Murine and human IgG isotypes, as well as Fc receptors, differ, complicating the determination of glycan-dependent antibody functions in vivo. IgG changes conformation when bound to the antigen, raising the possibility that studies using free monomeric antibody may not reflect IgG "in action." The two glycans within an IgG Fc region can be different, yielding pair effects that may have effector consequences distinct from those observed in homogeneously glycosylated IgG. Finally, IgG Fc glycosylation is a "zero sum game": an increase in the abundance of one glycoform necessarily comes at the expense of one or more others, leading to highly complex changes in the effector capacity of the IgG pool taken as a whole. Therefore, while it seems clear that changes in Fc glycans have consequences for humoral immune function, predicting and even measuring these effects has not proven straightforward.

In this review, we summarize variation in IgG Fc glycoforms over the lifespan, with a focus on the evidence that now establishes an unequivocal role for estrogens in these changes. We explore potential physiologic and pathophysiologic implications for this phenomenon, including in pregnancy and inflammatory diseases. Finally, we discuss candidate mechanisms by which estrogens could alter Fc glycans. Though much remains unknown, we suggest ways in which advancing techniques in glycobiology and next-generation sequencing will further deepen our understanding of developmental immunology.

11.2 IgG Fc Glycan Changes with Age, Sex, and Pregnancy

The relative abundance of glycan species within the IgG Fc region varies with age (Parekh et al. 1988a; Yamada et al. 1997). The largest adult series, assessing 5117 adults aged 18–95 across four populations, found a significant association with age for the large majority of IgG glycans (Kristic et al. 2014). The most consistent changes affect galactosylation, with the abundance of G0 forms increasing and of G2 forms decreasing progressively with age. Similar charges have been observed in other cohorts, with a decrease in sialylation accompanying the reduction in IgG Fc galactosylation (Yu et al. 2016; Bakovic et al. 2013). Pediatric series also reveal

age-dependent variation. Though the G1 fraction remains relatively stable at ~40–45% of all glycans throughout adulthood, this fraction is reduced to ~35% in the first few years of life, a period during which G2 glycoforms are somewhat more abundant (Parekh et al. 1988a; Shikata et al. 1998; Cheng et al. 2020). After these first few years, galactosylation rises modestly with age through childhood (the reverse of the adult trend) and fucosylation declines slightly (Cheng et al. 2020; Pucic et al. 2012; de Haan et al. 2016; Pezer et al. 2016; van Erp et al. 2020). In both adults and children, age-associated changes are sufficiently regular to enable approximate estimation of age from IgG glycans alone (Kristic et al. 2014; Cheng et al. 2020).

A consistent finding across many of these population studies is a shift in IgG glycans in females coincident with periods of hormonal transition (Yamada et al. 1997; Kristic et al. 2014; Bakovic et al. 2013; Shikata et al. 1998; Pucic et al. 2012; Knezevic et al. 2009; Chen et al. 2012; Ercan et al. 2017).

In particular, females of reproductive age demonstrate a greater proportion of galactosylated forms than males of the same age, with these sex-based trajectories converging rapidly around age 50, the typical age of menopause (Kristic et al. 2014; Bakovic et al. 2013; Ercan et al. 2017). A reciprocal shift is observed around the age of menarche, with females beginning to exhibit greater IgG galactosylation than males around age 12 years (Cheng et al. 2020; Pucic et al. 2012). Although females and males both undergo a so-called "mini-puberty" in infancy and early childhood, characterized by activation of the hypothalamic–pituitary–gonadal axis shortly after birth, no associated variation in IgG galactosylation is observed, potentially because sex hormone levels remain low (Cheng et al. 2020; Lanciotti et al. 2018; Bidlingmaier et al. 1973).

Changes accompanying major hormonal inflection points are especially intriguing in light of the similar IgG Fc glycan shifts observed during pregnancy. First reported in 1991, multiple studies have now confirmed that IgG Fc galactosylation and sialylation increase during pregnancy, peaking during the third trimester (Rook et al. 1991; Wuhrer et al. 2007; van de Geijn et al. 2009; Bondt et al. 2014). Many physiological adaptations coincide with these changes, among which is a nearly 20-fold rise in circulating estrogens (Abbassi-Ghanavati et al. 2009). These observations, along with the evidence suggesting an increase in galactosylation at menarche and a decrease at menopause, are consistent with the possibility that estrogens promote IgG Fc glycosylation.

11.3 Estrogens as the First Confirmed Modulators of the Human IgG Fc Glycome

In order of increasing potency, the three endogenous human estrogens are estriol (E3), estrone (E1), and 17(beta)-estradiol (E2). Estriol is produced almost exclusively during pregnancy by the placenta and is suspected to influence uteroplacental

blood flow and consequent cerebral development during fetal life. Estradiol is the major circulating estrogen from puberty to menopause, including in pregnancy. Estrone exists in equilibrium with estradiol via 17β-hydroxysteroid dehydrogenase and gradually becomes the only remaining estrogen as the ovaries cease to produce estradiol at menopause (Tepperman and Tepperman 1987). These molecules differ only by one hydroxyl or ketone moiety and bind the same estrogen receptors with affinities that correspond to their potencies (Fig. 11.1). Consequently, with the exception of subtle differences not further considered here, they exert highly similar biological effects and are collectively referred to as "estrogens."

To test whether female sex hormones directly regulate IgG Fc glycosylation, Ercan et al. quantitated the abundance of IgG-associated glycoforms in two populations of adult blood donors, confirming an increase in the abundance of G0 glycans in females at age 50 years, a difference that was further sharpened in 189 females in whom premenopausal vs. postmenopausal status was known by history (Ercan et al. 2017). The glycan shift was noted primarily as an increase in

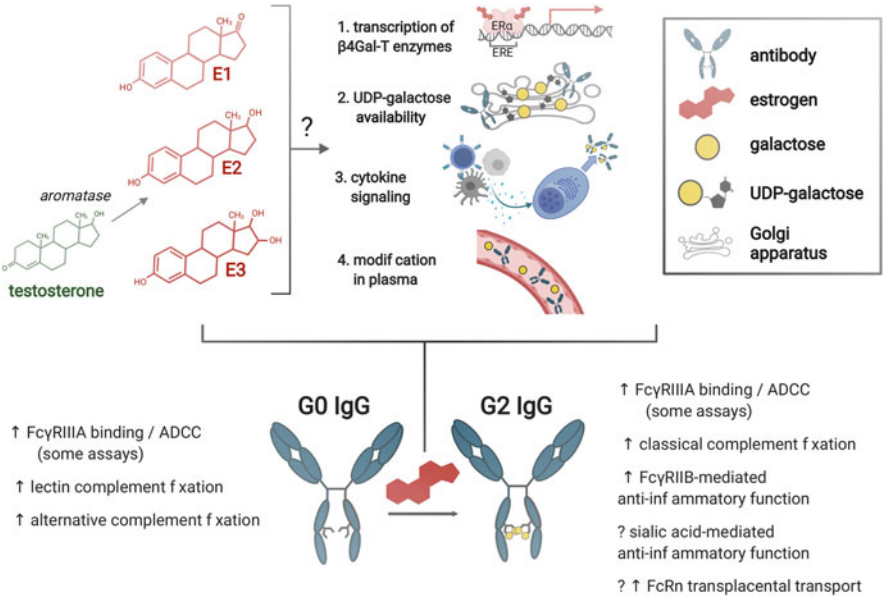

Fig. 11.1 IgG Fc glycan regulation by estrogens: potential mechanisms and functional implications. Estrogen occurs in three forms, estrone (E1), estradiol (E2), and estriol (E3), the latter produced principally from the placenta during pregnancy. Testosterone modulates IgG Fc glycans via interconversion to estradiol. Shown are four candidate mechanisms, not mutually exclusive, that may contribute to the effect of estrogens to increase the abundance of G2 IgG Fc glycans at the expense of G0 IgG Fc glycans. Potential implications of this shift for IgG effector functions are shown; note that enhanced FcγRIIIA binding is reported for both G0 and G2 glycans (please see text for discussion of function). *β4Gal-T* β4 galactosyltransferase, *ERα* estrogen receptor alpha, *ERE* estrogen response element, *UDP* uridine diphosphate, *ADCC* antibody-dependent cellular cytotoxicity, *FcRn* neonatal Fc receptor

the most abundant fucosylated non-bisected glycoform (G0F) but was much more modest in the bisected form G0FB.

To establish a causal role for estrogens, these investigators took advantage of several cohorts of human subjects undergoing hormonal manipulation. Van Pelt et al. randomized 119 healthy postmenopausal females to receive conjugated estrogens (a combination of estradiol, estrone, and equine estrogens), an estradiol mimetic (raloxifene), or placebo during a six-month exercise intervention (Van Pelt et al. 2014). Ercan et al. analyzed a random subset of these patients ($N = 58$) and found that, over an 18-month span, patients in the placebo arm exhibited a stable or slightly increased proportion of G0F IgG glycans, as expected given the known effect of age; by contrast, each estrogen arm exhibited a decrease of approximately 10% (Ercan et al. 2017). Shea et al. used leuprolide (an analog of gonadotropin-releasing hormone, GnRH) to suppress ovarian estrogen production in 70 healthy premenopausal females, with or without add-back transdermal estradiol for 5 months; both groups were then observed to the recovery of spontaneous menses (Shea et al. 2015). Analyzing a random subset ($N = 21$) of these patients, Ercan et al. found that the placebo group exhibited an immediate increase in IgG G0F glycans, a change not observed in the estrogen group. Both groups returned to pre-study G0F levels upon recovery from leuprolide (Ercan et al. 2017). Further analysis of samples from these premenopausal females confirmed that leuprolide changed the overall IgG glycan profile to resemble that of older females, but this change was fully prevented by estradiol (Juric et al. 2020). These studies establish a causal role for estrogens in the regulation of IgG Fc glycosylation in females, consistent with the direction and magnitude of the effects of menarche and menopause observed in population studies.

In males, a small amount of testosterone is routinely converted to estradiol by aromatase enzymes. To assess the effect of testosterone and estrogen on IgG glycans in males, Ercan et al. examined 40 patients from a cohort of males undergoing hormonal manipulation (Finkelstein et al. 2013). These subjects received the GnRH agonist goserelin acetate to suppress endogenous testosterone production, followed by replacement transdermal testosterone, placebo, or transdermal testosterone plus anastrozole (an aromatase inhibitor that blocks the conversion of testosterone to estradiol). Over a period of 12 weeks, males receiving testosterone exhibited no change in IgG glycans, whereas placebo subjects exhibited a significant increase in G0F glycans. Critically, patients are given testosterone plus anastrozole demonstrated a G0F increment identical to placebo, establishing that estrogens drive IgG Fc galactosylation also in males, albeit to a lesser degree than females because estrogen levels are substantially lower. By contrast, testosterone has no direct effect on IgG Fc glycosylation.

In a concordant study, Engdahl et al. characterized IgG Fc glycans in a cohort of 49 postmenopausal female rheumatoid arthritis (RA) patients randomized to hormone replacement therapy (in most patients, estradiol with the progestin norethindrone acetate) or no treatment. Patients receiving hormone supplementation demonstrated an increase in IgG Fc galactosylation as well as sialyation that inversely correlated with disease activity (Engdahl et al. 2018). It was not established

whether the increase in sialylated forms was a direct effect of hormone supplementation or instead mediated by the increase in galactosylation, a prerequisite to sialylation. The authors found that mRNA for ST6 β-galactoside α-2,6-sialyltransferase 1 (St6Gal-T1, encoded by *St6gal1*), an enzyme that adds sialic acid to IgG glycans, increased in response to estradiol in murine plasmablasts both in vitro and in vivo (mRNA and protein), and in human ex vivo-induced plasmablasts (mRNA only). Of note, mRNA for an enzyme that adds galactose to IgG glycans, β-1,4-galactosyltransferase 1 (β4Gal-T1), trended similarly but failed to achieve statistical significance in the sample size taken due to larger variance (Engdahl et al. 2018). Since galactosylated forms of IgG indeed increased in response to estradiol, it is unknown whether *St6gal1* transcription was induced directly by estrogen receptor activation or by some other regulator responsive to the increased galactosylated IgG substrate availability. Thus, these data are consistent with a direct effect of estrogens on IgG Fc sialylation but do not yet permit a firm conclusion.

With respect to galactosylation, these studies confirm that estrogens modulate IgG Fc glycans in humans, in females as well as males. Estrogens are thus the first and to date only mediator confirmed to have such an effect in vivo, driving the increase in IgG Fc galactosylation that accompanies menarche and disappears with menopause. Estrogens are likely also a major contributor to IgG glycan changes in pregnancy, though this connection has not been established experimentally. Testosterone plays no evident estrogen-independent role in the control of IgG Fc glycans. Effects of hormones such as progesterone and prolactin, or other factors associated with menarche, menopause, and pregnancy, remain to be determined.

11.4 Implications of Estrogen-Induced Fc Glycans Changes on IgG Function

Extrapolating from IgG Fc glycosylation changes to effector function is complex, as noted above. G0 glycoforms are typically regarded as rendering IgG pro-inflammatory, whereas G2 glycoforms and terminal sialic acid (obligatorily attached to galactose) are considered anti-inflammatory. Yet published findings conflict. G0 IgG activate complement via the mannose-binding lectin pathway and the alternative pathway (Malhotra et al. 1995; Arnold et al. 2006; Banda et al. 2008). Enzymatic removal of galactose enhances binding to FcγRIIIA and thus antibody-dependent cellular cytotoxicity (ADCC) (Ackerman et al. 2013). Conversely, G2 IgG engages immunoregulatory mechanisms by binding FcγRIIB, while terminal sialic acid is implicated in a range of anti-inflammatory and immunoregulatory functions (Karsten et al. 2012; Wang and Ravetch 2019). However, IgG Fc galactosylation can also be shown to enhance FcγRIIIA binding, ADCC, and complement fixation via the classical pathway (C1q), while effects of sialylation are not invariably observed (Dekkers et al. 2017; de Taeye et al. 2019; Jennewein

et al. 2019). Thus, no straightforward answer is yet available to the question of whether estrogen makes the overall pool of IgG more or less poised to promote inflammation.

The suggestion that G0 IgG is "net" pro-inflammatory comes principally from studies in inflammatory arthritis. Patients with RA and juvenile idiopathic arthritis (JIA) have long been recognized to exhibit an excess of G0 IgG, even preceding disease onset, and glycan aberrancy correlate with disease activity (Parekh et al. 1985, 1988b; Ercan et al. 2010, 2012a, b; Rombouts et al. 2015). G0 IgG appears especially pathogenic in murine models of antibody-mediated arthritis, while sialylation confers protection (Engdahl et al. 2018; Rademacher et al. 1994; Pfeifle et al. 2017). Human RA often improves transiently with pregnancy, only to flare after parturition, events that correlate with changes in IgG Fc galactosylation (Rook et al. 1991; van de Geijn et al. 2009; Bondt et al. 2013; Reiding et al. 2017). However, although RA incidence peaks in postmenopausal females, males also demonstrate a late peak, and hormone replacement therapy has no consistent effect on disease incidence or activity (Walitt et al. 2008; Hall et al. 1994). Of note, RA patients develop serologic evidence of inflammation well before clinical findings, and IgG Fc galactosylation varies with disease activity, rendering it very challenging to distinguish whether the relationship of IgG Fc glycan changes with disease activity represents one of cause, effect, or both (Ercan et al. 2012b; Deane et al. 2010).

More generally, females appear less susceptible to infection and more prone to autoimmunity, including autoantibody-driven conditions such as RA and systemic lupus erythematosus (SLE), compared to males (vom Steeg and Klein 2016; Libert et al. 2010). These differences integrate a host of immune and non-immune sexual dimorphisms, but would be unexpected if estrogens simply diminished IgG potency. Extrapolating from these observations, it is likely that the overall effects of estrogens on IgG potency will not be reflected accurately in a simple stronger/weaker dichotomy, but will likely vary with physiological context.

11.5 Implications of Estrogen-Induced Fc Glycan Changes for Pregnancy

The highest circulating estrogen concentrations in normal human physiology are attained during pregnancy. Elevated levels of estradiol and estriol are sustained for months, raising the possibility that pregnancy could be an important physiological context for estrogen-induced changes in IgG Fc glycans. A shift toward anti-inflammatory IgG Fc glycoforms, for example, could be an immune adaptation that helps maintain tolerance to the fetus, although no studies have yet tested this hypothesis.

One intriguing possibility is that estrogens regulate antibody transfer across the placenta. The central molecular mechanism mediating this transfer is the neonatal Fc

receptor (FcRn) (Roopenian and Akilesh 2007; Martinez et al. 2018). FcRn has a high affinity for the Fc portion of IgG at acidic pH (<6.5), but not at physiological pH (Roopenian and Akilesh 2007; Rodewald 1976). Syncytiotrophoblasts employ FcRn to capture IgG antibodies from maternal circulation in acidic endosomes and then deliver these vesicles to fetal endothelial cells, where physiological pH promotes dissociation of IgG from FcRn. IgG1 and IgG4 are transported with somewhat greater efficiency than IgG2 and IgG3 (Malek et al. 1996; Stapleton et al. 2011; Einarsdottir et al. 2014a, b). Much remains unknown about this important process, including whether antibodies against different targets equilibrate evenly across the placenta (Jennewein et al. 2019; Martinez et al. 2018, 2019; Fu et al. 2016).

As noted above, maternal IgG becomes more galactosylated as pregnancy progresses, likely due at least in part to estrogens. Intriguingly, comparison of total IgG from umbilical cord blood to maternal blood shows a further skew away from G0 and toward G2 glycoforms, suggesting selection for antibodies based on their glycosylation (termed a "placental sieve") (Jennewein et al. 2019; Williams et al. 1995; Kibe et al. 1996; Jansen et al. 2016). Relative to pre-term infants, antibodies in term infants are significantly more galactosylated, perhaps reflecting the cumulative effects of the sieve through gestation (Twisselmann et al. 2018). Proposed mechanisms underlying this skew include preferential affinity of G2 IgG for FcRn and possible engagement of the Fc receptors FcγRIIIa or FcγRIIb, also expressed at the maternal-fetal interface (Martinez et al. 2018, 2019; Kameda et al. 1991; Takizawa et al. 2005; Ishikawa et al. 2015; Mishima et al. 2007). These G2 antibodies may be especially well-suited to activate neonatal NK cells, providing enhanced immune defense to the fetus (Jennewein et al. 2019). In this manner, estrogen-driven enhancement of maternal IgG Fc galactosylation may set the stage for the provision of particularly protective antibodies for the fetus.

However, these observations remain controversial. The extent of G2 skewing is subtle, suggesting that any "sieve" is of low efficiency. IgG recognizing different antigens may exhibit differential glycan enrichment or lack enrichment altogether (Martinez et al. 2019). Further, when IgG Fc glycans are assessed in an isotype-specific manner, glycan-specific enrichment is largely absent, suggesting that the whole-IgG observations could reflect at least in part the known selectivity of FcRn for IgG1 (Malek et al. 1996; Stapleton et al. 2011; Einarsdottir et al. 2014a, b). In many experimental systems, Fc glycosylation has no impact on FcRn binding, and mice engineered to express human FcRn exhibit no differential IgG transfer to the fetus as a function of IgG Fc glycans (Liu et al. 2011; Souders et al. 2015; Cymer et al. 2017; Borghi et al. 2020). Thus, the extent to which estrogen-driven changes in IgG Fc galactosylation facilitate transplacental transfer remains uncertain. Indeed, the G2 skew of maternal IgG Fc glycans driven by estrogens in pregnancy renders a placental G2 "sieve" largely redundant. Rather, enhanced FcγRIIIa engagement by G2 IgG could still promote NK cell-mediated protection of the fetus and neonate, the role proposed for the sieve, providing an appealing teleological explanation for why estrogens regulate IgG Fc glycans at all (Dekkers et al. 2017; Jennewein et al. 2019; Kimura et al. 2000).

11.6 In Search of Mechanism

The molecular processes that link estradiol to IgG Fc glycan variation remain unknown. Presumably, the mechanisms responsible for these shifts must begin with an estrogen receptor, include a β4 galactosyltransferase, and potentially involve a sialyltransferase. There are seven β4 galactosyltransferase enzymes in humans (β4Gal-T (Sondermann et al. 2000; Barb and Prestegard 2011; Subedi and Barb 2015; Nose and Wigzell 1983; Parekh et al. 1988a; Yamada et al. 1997; Kristic et al. 2014), encoded by *B4GALT* (Sondermann et al. 2000; Barb and Prestegard 2011; Subedi and Barb 2015; Nose and Wigzell 1983; Parekh et al. 1988a; Yamada et al. 1997; Kristic et al. 2014)) with varying cellular localizations and substrate specificities. β4Gal-T1 has been shown to induce large changes in IgG Fc galactosylation in human cell lines, but the contribution of the other six galactosyltransferases has yet to be resolved (Kimura et al. 2000).

IgG N-glycan modification occurs predominantly in the Golgi apparatus. B cells express both estrogen receptors, ERα and ERβ, through many developmental stages (Kanda and Tamaki 1999) and this expression has been accompanied by estrogen responsivity in various forms, including increased production of IgM and IgG, reduced B cell receptor (BCR) signaling, and resistance to apoptosis (Kanda and Tamaki 1999; Grimaldi et al. 2002; Hill et al. 2011). Residing typically within the nucleus, ERα and ERβ act as transcription factors to change the expression of genes whose promoters are enriched for estrogen response elements (EREs). The genes of three enzymes that add galactose to glycan structures (β-1,4-galactosyltransferase 1, 3, and 7) contain an ERE in their promoter, but binding to ERα or Erβ has yet to be confirmed experimentally (Bourdeau et al. 2004).

In the absence of evidence for direct transcriptional regulation by estrogen, four distinct non-mutually-exclusive mechanisms exist whereby estrogen could enhance IgG Fc galactosylation (Sondermann et al. 2000). ERα and ERβ coordinate with some other transcription factor to increase the expression of a β4 galactosyltransferase in antibody-secreting B cells (Barb and Prestegard 2011); estrogens modulate the activity of a β4 galactosyltransferase via changes in substrate availability or product stability (Subedi and Barb 2015); changes in B cell galactosylation activity are mediated by cytokine signaling from other cells sensitive to estrogens (Nose and Wigzell 1983); or the effect of estrogens is independent of B cells altogether and is instead related to the plasma activity of β4 galactosyltransferases or differential lifespan of IgG Fc glycoforms in the plasma (Fig. 11.1).

11.6.1 Possibility 1: Transcriptional Activation of B4GALT Genes by Regulatory Complexes Containing ERα/ERβ

Some experimental and computational evidence has been gathered for the possibility that ERα and ERβ coordinate with some other transcription factor to increase the expression of a *B4GALT* gene. Multiple studies have found that ERα and ERβ form a complex with Sp1 (Porter et al. 1997; Saville et al. 2000; Bartella et al. 2012; Barreto-Andrade et al. 2018), a transcriptional activator of several *B4GALT* genes in certain lineages (Sato and Furukawa 2004; Jiang et al. 2006; Shen et al. 2007). Undirected high-throughput assays to detect protein–DNA interactions, on the other hand, suggest that the transcription factor ERRγ (estrogen-related receptor gamma, encoded by *ESRRG*, an orphan nuclear receptor named solely for sequence homology to the nuclear estrogen receptor) may mediate the connection between ERα and *B4GALT1*. HT-SELEX (high-throughput systematic evolution of ligands by exponential enrichment) of 984 human transcription factors reported interactions between ERRγ and the promoter of *B4GALT1*, as well as an interaction between *ESRRG* and ERα (Jolma et al. 2013). Integration of various high-throughput protein–DNA interactions in cell lines validated the ERRγ-*B4GALT1* interaction, further confirmed by homology-based molecular dynamics models (Kulakovskiy et al. 2013; Pujato et al. 2014). Direct experimental validation by antibody-secreting cells is needed to confirm this signal further and establish its role in estrogen-driven glycan changes.

11.6.2 Possibility 2: Modulation of β4 Galactosyltransferase Enzymatic Activity by Estradiol

The frequency of galactosylated glycans reflects not only the expression levels of *B4GALT* genes but also the availability of the galactose substrate. This substrate, uridine diphosphate (UDP)-galactose, is formed in the cytosol when galactose-1-phosphate uridyltransferase (GALT) exchanges the phosphate on a galactose molecule phosphorylated by galactokinase (GALK) for UDP (Slepak et al. 2005). In the cytosol, UDP-galactose 4′-epimerase (GALE) maintains a balance between UDP-galactose and UDP-glucose through reversible epimerization (Seo et al. 2019; Broussard et al. 2020). UDP-galactose translocator enzymes then deliver UDP-galactose to the Golgi lumen against a severe UDP-galactose concentration gradient, anti-porting uridine monophosphate (UMP^{2-}) to leverage the acidity of the cytosol relative to the Golgi compartment (Parker and Newstead 2019). Thus, substrate availability for the β4 galactosyltransferase enzymes is dependent on the activity of GALK, GALT, GALE, and UDP-galactose transporters, as well as the relative concentrations of UMP^{2-} and H^+ between the Golgi apparatus and the cytosol. Evidence for ERα/β-driven changes in these factors is limited, since the hypothesis that estrogens modulate the availability of substrate for galactosyltransferases has not been specifically investigated. Homology-based

molecular dynamics models have suggested that *SLC35E3*, an orphan translocator with marked sequence homology to Golgi membrane nucleotide sugar transporters, is a direct target of ERα (Pujato et al. 2014). More intriguingly, high-throughput DNA–protein interaction assays have reported that ERRγ binds the promoters for *GALT* and *GALE* in addition to *B4GALT1*, furthering the evidence that it will be fruitful to investigate this transcription factor (Jolma et al. 2013; Kulakovskiy et al. 2013). In RA, some studies but not others have found reduced activity of β4 galactosyltransferases (Furukawa et al. 1990; Axford et al. 1987; Jeddi et al. 1996). Of note, IgG Fc galactosylation is a reversible reaction, susceptible to hydrolysis of galactose by the enzyme β-galactosidase. Elevated activity of this enzyme has been reported in RA; regulation by estrogen has not been explored (Su et al. 2020).

11.6.3 Possibility 3: Secondary Cytokine Signaling

Many cells express ERα and ERβ, raising the possibility that the effect of estrogens on IgG is mediated indirectly via mediators produced by other lineages. For example, in cultured B cells, IgG Fc galactosylation is augmented by interleukin 21 (IL-21) and decreased by all-trans retinoic acid (Wang et al. 2011). The enormous number of potentially relevant cell–cell interactions render this question well-suited for genome-wide association studies (GWAS), which employ inter-individual genetic variation to probe many biological hypotheses in parallel. Lauc et al. were the first to study the frequency of IgG glycoforms as quantitative traits, querying for associated variation with 296,619 single-nucleotide polymorphisms (SNPs) across the genome (Lauc et al. 2013). In the discovery cohort (four European populations, $N = 2247$), the authors observed nine genome-wide significant associations between genetic loci and IgG Fc glycan traits. For galactosylation, these loci included a region containing *B4GALT1* and a second region containing the genes *ANKRD55* and *IL6ST*. While the function of *ANKRD55* is undefined, *IL6ST* encodes a subunit of several cytokine receptors, including the interleukin 6 (IL-6) receptor. SNPs in the *ANKRD55-IL6ST* locus exhibited opposite effects on the frequency of G0 and G2 IgG glycans, consistent with an underlying change in the net galactosylation rate. Though the lead *ANKRD55-IL6ST* SNP failed to replicate in a second cohort ($N = 1848$) with respect to G2 frequency ($p = 0.6$) and reached only nominal significance ($p = 0.01$) for G0 frequency, this could potentially be an artifact of population stratification or differential glycoform ascertainment in ultra-performance liquid chromatography (UPLC) compared to mass spectrometry (MS).

Several GWAS have since extended the approach put forward by Lauc et al., each replicating the expected association with *B4GALT1* (Shen et al. 2017; Wahl et al. 2018; Klaric et al. 2020). Shen et al. reanalyzed a subset of the individuals in Lauc et al., but in recognition of the inherent correlation structure between glycoform frequencies, used multivariate analysis of variance (MANOVA) to uncover SNPs explaining variance in the overall distribution of IgG glycoforms (Shen et al. 2017).

Despite this promising methodological approach, these analyses were not suited to find specific galactosylation regulators because glycoform variations were modeled *within* each galactosylation state rather than between them (the distribution of G0F, G0N, and G0, for example, rather than the distribution of G0F, G1F, and G2F). Wahl et al. examined MS data in a separate cohort ($N = 1823$) and noted several associations between *RUNX3*, a transcription factor frequently silenced in cancer, and galactosylation and bisection glycoforms from IgG2 (Wahl et al. 2018; Bae and Choi 2004). With the largest IgG glycome GWAS to date ($N = 8090$), Klarić et al. clarified that the *RUNX3* glycan phenotype effect size distribution resembled that of enzymes known to induce bisection rather than enzymes known to induce galactosylation (Klaric et al. 2020). This study also uncovered and replicated an association between the frequency of digalactosylated glycoforms and *HIVEP2*, encoding a zinc finger transcription factor that has been implicated in T cell activation (Nomura et al. 1991). It has been noted that the expression of this transcription factor is reduced in breast tumor samples, but this appears to be independent of estrogen receptor expression, and we are unaware of any other *HIVEP2* connections to estrogen biology (Fujii et al. 2005). While Klarić et al. replicated the *ANKRD55-IL6ST* signal with nearly genome-wide significance ($P = 7.22 \times 10^{-6}$), Wahl et al. did not, perhaps due to their conservative Bonferroni correction that assumed 50 independent tests (given the unique correlation structure of glycome phenotypes, robust statistical inference requires multivariate models or permutation simulations to uncover the true number of independent tests).

The evidence for a role of IL-6 in multiple cohorts is intriguing, given that estrogen negatively regulates IL-6 signaling in many cells, including those in the B cell lineage (Pottratz et al. 1994; Stein and Yang 1995; Kurebayashi et al. 1997; Liu et al. 2005; Canellada et al. 2008). These observations raise the possibility that estrogens modulate IgG Fc galactosylation in part through interference with IL-6 production or signaling, a pathway exploited by therapeutic blockade of IL-6 in RA (Emery et al. 2008). Whether IL-6 in fact modulates IgG Fc galactosylation, and which other mediators also participate in this regulation, are outstanding questions that should be interrogated experimentally.

11.6.4 Possibility 4: Post-Secretory Glycan Modification

A final possibility is that estrogen regulation of IgG Fc glycans bypasses B cells entirely. Considering the IgG lifespan in three phases—generation, circulation, and degradation—brings to light the possibility that estrogens might modify IgG Fc glycans after release from B cells. Recent studies have shown that enzymes capable of modifying IgG Fc galactose and sialic acid content are present and active in the plasma (Wandall et al. 2012; Jones et al. 2016; Catera et al. 2016). ST6Gal-T1, for example, is released by the liver as an acute phase reactant (Dalziel et al. 1999). Conditional knock-out mouse models demonstrate that IgG sialylation can occur even when expression of *St6gal1* by B cells has been ablated, though follow-up work

suggests that such effects are modest (Jones et al. 2016; Schaffert et al. 2019). Whether estrogens modulate post-secretory changes in IgG glycans remains unknown, although some evidence implicates estrogens in the activation of platelets, which are known to release soluble β4Gal-T1 along with sufficient levels of sugar nucleotides to galactosylate exogenous substances (Maccarrone et al. 2002; Moro et al. 2005). We are unaware of any data implicating estrogens in antibody degradation and whether it may preferentially do so for certain glycoforms.

11.7 Future Directions

The promotion of IgG Fc galactosylation by estrogens represents a new link between sex and immunity, adding a contextual layer that brings us closer to understanding situationally appropriate immune reactivity over the lifespan (Fig. 11.1). The physiological consequences of this estrogen effect remain undefined. The especially marked changes in IgG Fc glycans during pregnancy, presumably related at least in part to high and sustained levels of estrogens, favor a role in the protection of the fetus and/or mother. The mechanisms mediating estrogen-driven IgG galactosylation remain unknown, requiring experiments to clarify whether the estrogen effect is mediated directly by B cells expressing estrogen receptors or via other pathways. Convergent studies highlight potential roles for Sp1, ERRγ, and IL-6 signaling, justifying targeted experimental follow-up.

It remains unknown whether the effect of estrogens on glycans is unique to the IgG Fc region or occurs more broadly among N-glycosylated proteins, including in other types of antibody. Menarche- and menopause-related changes in whole-plasma glycans are comparable, if somewhat less marked than those found in IgG (Pucic et al. 2012; Knezevic et al. 2009, 2010). Since whole-plasma samples contain IgG-derived glycans, it is difficult to determine if such changes arise from any proteins beyond IgG. Nonetheless, glycoform shifts with age, in pregnancy, and in arthritis and other autoimmune diseases highlight the importance of IgG Fc glycans as an axis of immunoregulation (Cheng et al. 2020). Ultimately, decoding the pathways that define the IgG Fc glycome will provide a much higher resolution view of immune development and attendant opportunities for therapeutic intervention.

Compliance with Ethical Standards

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Part III
Effector Functions and Diseases

Chapter 12
Sweet Rules: Linking Glycosylation to Antibody Function

Falk Nimmerjahn and Anja Werner

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Abstract Antibodies produced upon infections with pathogenic microorganisms are essential for clearing primary infections and for providing the host with long-lasting immunity. Moreover, antibodies have become the most widely used platform for developing novel therapies against cancer and autoimmunity, requiring an in-depth understanding of how antibodies mediate their activity in vivo and which factors modulate pro- or anti-inflammatory antibody activities. Since the discovery that select residues present in the sugar domain attached to the immunoglobulin G (IgG) fragment crystallizable (Fc) region can modulate both, pro- and anti-inflammatory effector functions, a wealth of studies has focused on understanding how IgG glycosylation is regulated and how this knowledge can be used to optimize

F. Nimmerjahn (✉)
Chair of Genetics, Department of Biology, Institute of Genetics, University of Erlangen-Nürnberg, Erlangen, Germany

Medical Immunology Campus Erlangen, Erlangen, Germany
e-mail: falk.nimmerjahn@fau.de

A. Werner
Chair of Genetics, Department of Biology, Institute of Genetics, University of Erlangen-Nürnberg, Erlangen, Germany

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therapeutic antibody activity. With the introduction of glycoengineered afucosylated antibodies in cancer therapy and the initiation of clinical testing of highly sialylated anti-inflammatory antibodies the proof-of-concept that understanding antibody glycosylation can lead to clinical innovation has been provided. The focus of this review is to summarize recent insights into how antibody glycosylation is regulated in vivo and how select sugar residues impact IgG function.

Keywords B cells · Antibody glycosylation · Therapeutic antibodies · Fc-receptors

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
Asp	Asparagine
B4GALT1	β-1,4-galactosyltransferase 1
BCR	B cell receptor
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CMP-SA	Cytidine monophospho-sialic acid
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcαR	Fc-alpha receptor
FcεR	Fc-epsilon receptor
FcγR	Fc gamma receptor
FcRn	Neonatal Fc receptor
FNAIT	Fetal or neonatal alloimmune thrombocytopenia
G0	Agalactosylated
G1	Monogalactosylated
G2	Digalactosylated
GlcNAc	*N*-acetylglucosamine
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IVIg	Intravenous immunoglobulin G
JC	Joining chain
K_A	Association constant
Man	Mannose
MBL	Mannose-binding lectin
MHCII	Major histocompatibility complex II
N	Asparagine
NK cell	Natural killer cell

PC	Plasma cell
PNGaseF	Peptide-*N*4-(*N*-acetyl-beta-glucosaminyl) asparagine amidase
RA	Rheumatoid arthritis
SC	Secretory component
ST6Gal1	β-galactoside-α2,6-sialyltransferase 1
STAT6	Signal transducer and activator of transcription 6
TCR	T cell receptor
TD	T cell-dependent
Tfh cell	T follicular helper cell
Th cell	T helper cell
TI	T cell-independent
TNFα	Tumor necrosis factor-alpha
Treg	Regulatory T cell

12.1 Introduction

With the introduction of second-generation glycoengineered therapeutic antibodies optimized for Fc receptor binding into clinical application, the field of antibody glycosylation has shifted from a mere descriptive, method oriented research area to a highly translational field with proven clinical impact (Li et al. 2017b; Yu et al. 2017). More recently, highly sialylated immunomodulatory IgG preparations have successfully passed first clinical test phases, emphasizing that a detailed understanding of the molecular and cellular pathways modulating antibody glycosylation can drive clinical innovation (Arroyo et al. 2019; Seeling et al. 2017). All human and mouse antibody isotypes are characterized by specific sugar moieties attached to the antibody fragment crystallizable (Fc) domain (Fig. 12.1). While IgM, IgA, and IgE isotypes have several N-linked glycosylation sites in the Fc fragment, immunoglobulin G (IgG) is characterized by only one sugar moiety attached to Asn297 of each CH2 domain (Arnold et al. 2007). In addition to N-linked sugar moieties, IgA and certain other immunoglobulin isotypes and subclasses may also contain highly complex O-linked sugar residues (Arnold et al. 2007). Although the existence of these sugar residues has been known for decades, a more in-depth understanding of the effects these post-translational modifications may have on antibody activity have only been obtained during the last ten to fifteen years. Due to the highly complex nature of the multiple sugar domains present in IgM, IgA, and IgE, an initial focus of the research was to study the single sugar domain present in each of the two IgG heavy chains. Despite the seemingly simple structure of this branched sugar domain, containing a heptameric core structure of *N*-acetylglucosamine (GlcNAc) and mannose (Man) residues, the variable addition of terminal galactose and sialic acid residues in combination with branched fucose and GlcNAc residues can create a high level of variability. Indeed, more than 30 different IgG glycosylation variants may be generated in principle, when anticipating that both sugar moieties within one

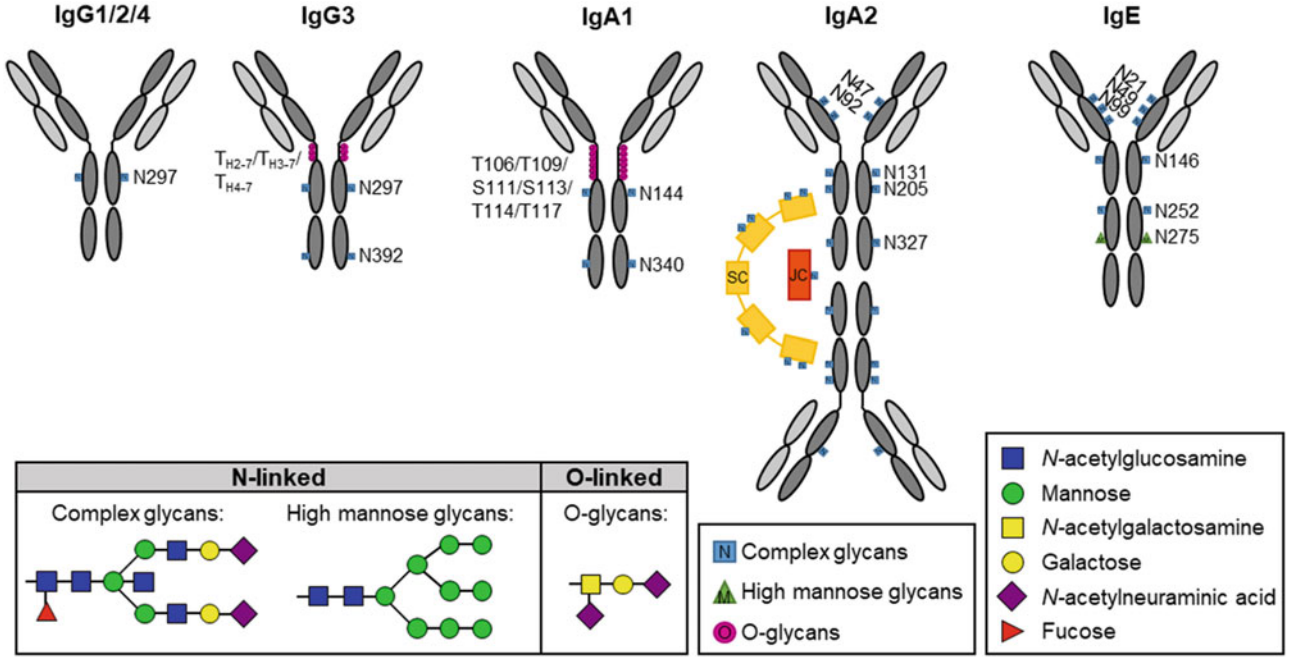

Fig. 12.1 Glycosylation sites and sugar moieties of antibody isotypes. Schematic representation of the glycosylation sites in IgG, IgA, and IgE molecules. The glycosylation sites for IgG are indicated by their amino acid number according to Arnold et al. (2007) and Plomp et al. (2016), while the other isotypes follow UniProt numbering. Each immunoglobulin consists of two heavy (dark gray) and two light chains (light gray), with IgA1 forming dimers complexed with the joining chain (JC; orange) and the secretory component (SC; yellow). N-linked glycosylation sites are divided into complex and high mannose glycans depicted by a blue square and a green triangle, respectively. Furthermore, O-linked glycans are illustrated by a purple circle. Schematic representative glycan structures for N-linked complex and high mannose glycans, as well as O-linked glycans, are depicted

IgG molecule are the same (Arnold et al. 2007; Kobata 2008; Raju 2008); if the individual IgG heavy chains contain different sugar structures, theoretically more than 500 IgG glycosylation variants may be generated (Holland et al. 2006; Masuda et al. 2000; Saphire et al. 2002). Potentially limiting the overall complexity, recent reports suggest that individual IgG subclasses may have distinct glycosylation patterns (de Haan et al. 2017; Kao et al. 2017; Wuhrer et al. 2007). In addition to the Fc-linked sugar moiety, some IgG antibodies may also have a sugar domain attached to the antibody variable domain (Fragment antigen binding, Fab). The glycosylation acceptor site for such sugar domains is not encoded in the germline but rather generated de novo during the process of antibody hypermutation (van de Bovenkamp et al. 2018). Indeed, it was suggested that Fab-linked sugar moieties play an important role in antigen recognition. In contrast to Fc-linked sugar domains, Fab-associated sugar structures are characterized by a higher level of fully processed sugar moieties, rich in terminal galactose and sialic acid residues. In this review, we will focus on the function of Fc-linked sugar structures, and the interested reader is directed to the excellent recent review article on Fab glycosylation (van de Bovenkamp et al. 2018).

12.2 Regulation of Antibody Glycosylation

Glycan biosynthesis takes place in the endoplasmic reticulum (ER) and the Golgi apparatus of B cells (van Kooyk et al. 2013). After an initial synthesis of high mannose N-glycans in the ER, proteins are transferred to the Golgi, where the trimming and addition of further sugar moieties are catalyzed by different glycosyltransferases, which are differentially expressed within the Golgi (Boune et al. 2020; van Kooyk et al. 2013). Their activity can be dependent on, for example, transcription factors (Klaric et al. 2020; Rajput et al. 1996), availability of sugar donors (Milewski et al. 1991) or cytokines, like tumor necrosis factor-alpha (TNFα) (Garcia-Vallejo et al. 2005; Gringhuis et al. 2005). Thus, the ultimate glycan structure of glycoproteins like IgG is largely determined by the selective activity of glycosidases and glycosyltransferases as well as by individual metabolic conditions within the cell, enabling to adopt IgG glycosylation to altered environmental conditions (Wang et al. 2011). In addition to B cell-intrinsic antibody glycosylation, it was recently proposed that IgG sialylation might occur independently of the secretory pathway allowing a B cell-independent modification of IgG glycosylation (Dougher et al. 2017; Jones et al. 2016; Lee et al. 2014; Manhardt et al. 2017). With respect to the use of therapeutic antibodies, this would pose a major problem, as such B cell-independent modifications of IgG glycosylation occurring upon IgG injection may alter IgG function in vivo. Mechanistically, it was suggested that soluble sialyltransferases may be generated by proteolytic cleavage of the membrane-associated enzymes in liver hepatocytes or platelets. Upon release into the circulation, CMP-sialic acid (CMP-SA) precursors may become conjugated to existing IgG sugar structures extracellularly (Lee et al. 2014; Manhardt et al. 2017; Wandall et al.

2012). As the appropriate sugar donors are not believed to be present in the circulation at sufficient amounts, however, it was proposed that degranulating platelets may provide CMP-SA, allowing for a B cell-extrinsic IgG glycosylation. Indeed, thrombin-activated platelets were shown to efficiently substitute for synthetic CMP-SA in providing sugar donors for extracellular β-galactoside-α2,6-sialyltransferase 1 (ST6Gal1)-mediated IgG sialylation (Lee et al. 2014). However, recent studies suggest that the efficiency of this B cell-extrinsic IgG sialylation pathway compared to B cell-intrinsic IgG sialylation may be very low. Thus, no major increase in serum IgG sialylation was detectable in mice with a B cell-specific knockout of ST6Gal1 (Ohmi et al. 2016). Furthermore, the injection of desialylated IgG into B cell-deficient mice (lacking B cell-intrinsic but not extrinsic IgG sialylation pathways) revealed that B cell-extrinsic IgG glycosylation occurs at very low levels and predominantly in the more easily accessible Fab fragment of the IgG molecule (Schaffert et al. 2019).

The general idea that antibody glycosylation may impact its function comes from the finding that antibody glycosylation is not stable, but can change upon immune stimulation. It has been firmly established, for example, that during microbial infection, vaccination, autoimmune inflammation, aging, and pregnancy characteristic changes in serum IgG glycosylation affecting most dominantly terminal sugar residues can occur (Bartsch et al. 2020; de Haan et al. 2016; Jansen et al. 2016; Kaneko et al. 2006; Kao et al. 2017; Kristic et al. 2014; Mahan et al. 2016; Mehta et al. 2008; Moore et al. 2005; Parekh et al. 1985, 1988; Pezer et al. 2016; Pucic et al. 2012; Selman et al. 2012; Twisselmann et al. 2018; Vadrevu et al. 2018). Further along these lines, it was demonstrated that socio-economic factors might impact antibody glycosylation (Mahan et al. 2016; Stambuk et al. 2020). For example, it was shown that the serum IgG glycome in individuals living in developing countries, like Africa, was characterized by glycan traits with high levels of G0 glycan forms lacking terminal galactose and sialic acid residues. In contrast, in industrial countries having well-developed health systems and sufficient access to water and food, IgG glycan traits with higher levels of galactosylated and sialylated glycan species were observed (Mahan et al. 2016; Stambuk et al. 2020). In addition to the more frequent alterations in antibody sialylation and galactosylation, IgG core fucosylation, which is highly abundant in IgG sugar domains, can be strongly reduced on platelet specific alloantibodies in a disease called fetal or neonatal alloimmune thrombocytopenia (FNAIT) (Kapur et al. 2014; Sonneveld et al. 2016). Moreover, low levels of IgG fucosylation were detected on Dengue virus-specific antibodies and correlated with more severe infections (Wang et al. 2017).

In mice, vaccination studies have provided valuable insights into how different pathways of B cell activation can impact IgG glycan composition. While immunization with T cell-independent (TI) antigens or T cell-dependent (TD) antigens without adjuvants mostly led to the formation of sialylated IgG molecules, stimulation with TD antigens under inflammatory conditions induced IgG antibodies with low levels of sialic acid residues (Hess et al. 2013; Oefner et al. 2012) (Fig. 12.2a). Whereas many studies were investigating global changes in IgG glycosylation, more recent reports investigated how individual IgG subclass glycosylation was affected

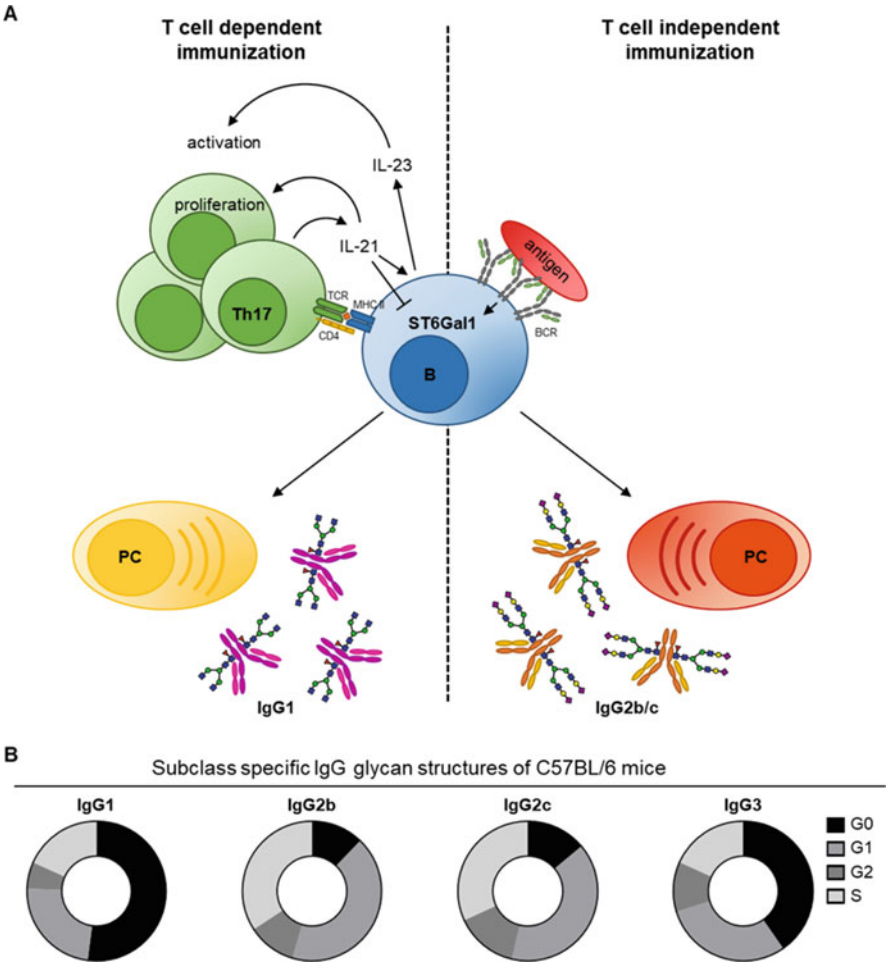

Fig. 12.2 Impact of immunization and IgG subclass on antibody glycosylation. (**a**) *Left:* Interaction between the T cell receptor (TCR) on T helper 17 cells (Th17) and major histocompatibility complex II (MHC II) on B cells (B) under pro-inflammatory conditions leads to the release of Interleukin 21 (IL-21) by Th17 cells which in turn induces Th17 proliferation and IL-23 production of B cells leading to T cell activation. Furthermore, T cell-dependent stimulation and IL-21 inhibit the β-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal1), inducing the generation of non-sialylated IgG1 antibodies by plasma cells (PC). *Right:* Crosslinking of the B cell receptor (BCR) by an antigen without adjuvants induces ST6Gal1 activation leading to the production of sialylated IgG antibodies. (**b**) IgG subclass-specific Fc glycosylation patterns in C57BL/6 mice. Depicted is the proportion of agalactosylated (G0), monogalactosylated (G1), digalactosylated (G2), and sialylated glycan species of the different IgG subclasses IgG1, IgG2b, IgG2c, and IgG3

upon vaccination. Interestingly, IgG subclass-specific changes were observed after stimulation with TI and TD antigens (Kao et al. 2017). Of note, IgG subclass-specific differences in glycosylation have already been noted under steady-state conditions

(de Haan et al. 2017; Kao et al. 2017). While murine IgG1 molecules were largely present in the G0 glycoform, IgG2a/c and IgG2b associated sugar moieties contained mono- or digalactosylated glycan structures with additional terminal sialic acid residues (Fig. 12.2b) (Kao et al. 2017). While it is difficult to assess the steady-state IgG subclass glycosylation in humans due to the constant exposure to immune stimulants (infections, vaccination), it was suggested that human IgG1 has higher levels of galactose containing sugar structures than IgG2 and IgG4 (Plomp et al. 2017). In addition, serum IgG3 seemed to contain more sialylated sugar domains compared to the IgG1 subclass (Sonneveld et al. 2018). As human IgG3 (and IgG1) functionally behaves very equal to mouse IgG2a/b/c subclasses, this result would be in line with the mouse IgG subclass glycosylation data. These results suggest that either IgG subclass structure or genetic factors linking IgG class switch to glycosylation play a major role in determining the precise composition of IgG subclass glycosylation.

Moreover, there is accumulating evidence that the type of T helper cell response—namely Th1, Th2, or Th17—can impose a distinct IgG glycosylation pattern. For example, B cells treated with Interleukin-21 (IL-21) and CpG significantly increased the generation of galactosylated and sialylated N-glycan species while decreasing bisecting GlcNAc (Wang et al. 2011). However, IL-21 in the context of Th17 cells and under pathogenic conditions seems to have the opposite effect on IgG glycosylation (Hess et al. 2013; Pfeifle et al. 2017). Thus, in a mouse model of rheumatoid arthritis (RA) IL-21 triggered the downregulation of ST6Gal1 expression in newly developing plasma blasts and plasma cells via IL-23-activated Th17 cells (Pfeifle et al. 2017). The IL-23-Th17 axis, which has already been associated with the pathogenesis of RA before (Lubberts 2015), seems to be responsible to control the intrinsic inflammatory activity of autoantibodies by shifting IgG glycosylation to pro-inflammatory sugar structures, triggering the clinical onset of autoimmune arthritis (Pfeifle et al. 2017). Moreover, IL-21 may act in an autocrine loop specifically supporting the proliferation and differentiation of Th17 cells (Korn et al. 2007). Furthermore, IL-21 may induce IL-23 production by B cells, further enhancing the autoimmune process by a positive feedback mechanism (Lee et al. 2015). The pivotal role of T follicular helper 17 (Tfh17) cells in modulating IgG sialylation was confirmed recently by showing that Tfh17 induction by IL-6 and IL-23 leads to a downregulation of ST6Gal1 allowing the production of desialylated pro-inflammatory IgG molecules (Bartsch et al. 2020). A similar inhibition of ST6Gal1 expression in germinal center B cells was also noted for water-in-oil adjuvants, which trigger IFNγ-producing Tfh1 cells (Riteau et al. 2016). Of note, not only IgG glycosylation, but also Fc-gamma receptor (FcγR) expression and IgG class-switching can be regulated via cytokines, creating complex regulatory networks, which need to be fine-tuned to ensure optimal antibody activity.

12.3 Impact of Glycosylation on IgG Activity

Before we discuss the impact of individual sugar residues on IgG activity in more detail, it is helpful to provide a short overview of the family of Fcγ-receptors (FcγRs). Fcγ-receptors bind the IgG Fc fragment near the hinge-proximal region in a 1:1 complex and include several activating FcγRs (FcγRI, FcγRIII, FcγRIV in mice and FcγRIA, FcγRIIA, FcγRIIC, FcγRIIIA, FcγRIIIB in humans) and one inhibitory FcγR (FcγRIIB) (Sondermann et al. 2000). Activating FcγRs are associated with a dimer of the common FcRγ chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM), while the inhibitory FcγRIIB transmits its signals via an immunoreceptor tyrosine-based inhibitory motif (ITIM) present in its cytoplasmic region (Fig. 12.3a) (Brandsma et al. 2016; Nimmerjahn and Ravetch 2008). As activatory and inhibitory type I FcγRs are usually co-expressed on the same cell, a balanced immune response can be triggered. Apart from FcγR expression level, the different affinities of individual IgG subclasses to select FcγRs to modulate the magnitude of downstream effector pathways. For example, the mouse and human high-affinity receptor FcγRI binds IgG2a (in mice) or IgG1 and IgG3 (in humans) with an affinity of 10^7–10^8 M^{-1} while most of the other FcγRs have a 100–1000-fold lower affinity for IgG subclasses (Fig. 12.3a). Furthermore, mouse IgG1 and IgG2a/c or IgG2b bind differentially to activating and inhibitory FcγRs, suggesting that IgG antibodies are subject to a subclass-specific negative regulation via the inhibitory FcγRIIB (Nimmerjahn and Ravetch 2005). For example, IgG1 binds with an approximately tenfold higher affinity to the inhibitory FcγRIIB compared to the binding to its activating receptor, FcγRIII. In contrast, IgG2a/c or IgG2b has a higher affinity for activating FcγRs, FcγRI and FcγRIV, resulting in a lower threshold for cell activation (Bruhns and Jonsson 2015; Nimmerjahn and Ravetch 2005; Takai 2005). To account for these differences in affinity and use them as a predictive tool for IgG subclass activity in vivo, the concept of the A/I ratio, in which the affinity of an individual subclass for the activating receptor is divided by the affinity for the inhibitory FcγRIIB, has been developed and was validated by bioinformatic modeling more recently (Nimmerjahn and Ravetch 2005; Robinett et al. 2018).

As mentioned before, changes in IgG glycosylation can alter IgG affinity (and hence the A/I ratio), allowing to use of this concept for assessing the potential impact of alterations in IgG affinity on IgG activity in vivo. In general, IgG glycosylation is critical to allow functional binding of the IgG-Fc domain to Fc receptors (Arnold et al. 2007; Nimmerjahn and Ravetch 2006, 2008; Walker et al. 1989). Crystal structural analysis revealed that the glycosylated horseshoe-shaped IgG Fc fragment can occur in an open or closed conformation while aglycosylated human Fc fragments seemed to predominantly exist in the closed IgG-Fc conformation, prohibiting functional Fc receptor binding (Ahmed et al. 2014; Borrok et al. 2012; Jefferis and Lund 1997; Krapp et al. 2003). The open conformation is probably supported by hydrophobic interactions between the two mannose residues in the α1,3-branch of the two oligosaccharides (Krapp et al. 2003; Raju 2008). Interestingly, individual

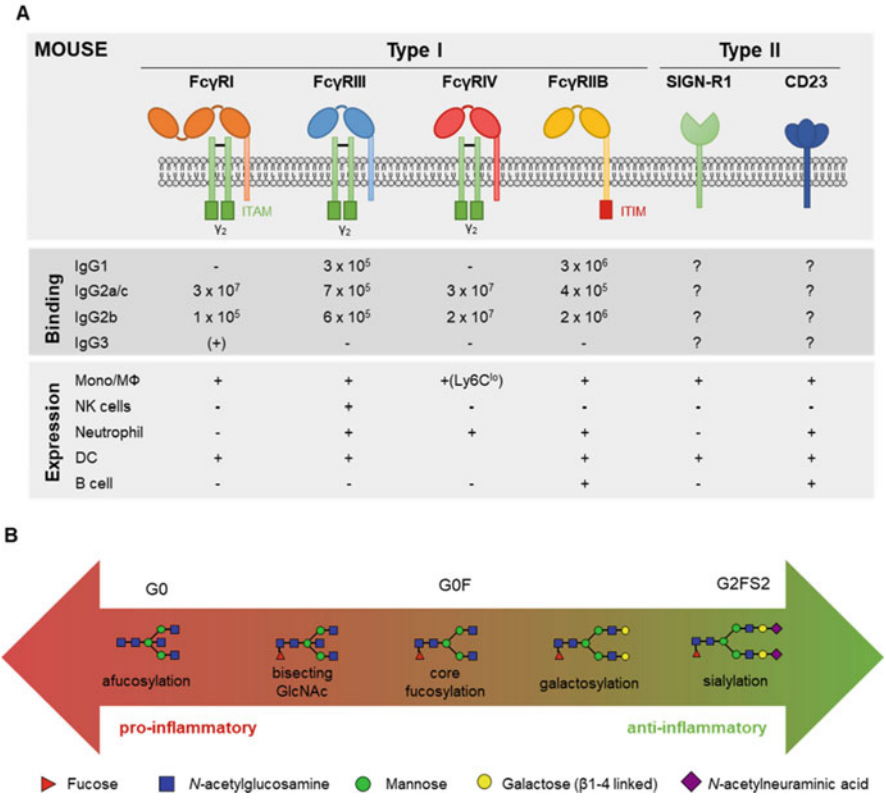

Fig. 12.3 IgG binding to FcγRs and impact of glycosylation on IgG activity. (**a**) *Upper part:* Schematic representations of murine Type I and Type II FcRs with associated gamma chain (γ_2) and immunoreceptor tyrosine-based inhibitory or activation motif (ITIM or ITAM). *Middle part:* Binding affinities of different murine IgG subclasses to individual Type I and Type II Fc receptors given as K_A (M^{-1}). -: no binding, (+): possible binding, ?: no reported affinity values. *Lower part:* Expression profile of individual FcRs on different immune cells. +: expression, -: no expression, +(Ly6C^{lo}): expression only on Ly6C^{lo} monocytes. (**b**) Impact of different IgG glycosylation patterns on antibody activity. Depending on the Fc glycan composition IgG molecules exhibit either more pro- or anti-inflammatory effector functions. Representative glycan structures associated with different IgG activities are depicted

IgG subclasses seem to have a differential requirement for the length of the N297-linked sugar moiety. While a single GlcNAc residue was sufficient to maintain the activity of mouse IgG2a and human IgG1 subclasses, trimming down the Fc-linked sugar moiety to one residue largely abrogated the activity of mouse IgG1 and IgG2b subclasses (Kao et al. 2015). Moreover, the four murine IgG subclasses (IgG1, IgG2b, IgG2a/c, and IgG3) are differentially glycosylated with 50 % of IgG1 being present in the G0 form, while IgG2b and IgG2a/c molecules show an increased proportion of galactosylated and sialylated glycan species (Fig. 12.2b), creating a complex situation when trying to decipher the contribution of individual sugar

moieties on IgG function. Yet another factor that is rarely considered when discussing the impact of IgG glycosylation on IgG function is the significant impact of higher order immune complexes, the natural ligand of the low-affinity FcγRs, may have. Thus, even aglycosylated IgG immune complexes demonstrated residual binding to the high-affinity FcγRI (Lux et al. 2013).

12.3.1 Impact of Sialic Acid on IgG Dependent Effector Functions

With respect to terminal sialic acid residues, several groups observed that high levels of IgG sialylation reduce antibody-dependent effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and complement-dependent cytotoxicity (CDC) (Kaneko et al. 2006; Li et al. 2017a; Quast et al. 2015; Scallon et al. 2007). Mechanistically, it was suggested that a lower level of binding of sialylated IgG variants to mouse and human activating FcγRs expressed on natural killer (NK) cells or myeloid cells may explain this reduced in vivo activity. In line with such a model, a change in IgG-Fc structure towards a more closed conformation was observed when high levels of terminal sialic acid residues were present in the IgG Fc-sugar structure, possibly explaining the reduction in binding to FcγRs (Ahmed et al. 2014; Sondermann et al. 2013). However, other studies found no major impact of sialylation on IgG structure (Crispin et al. 2013) and either no (Thomann et al. 2015) or even an increased activity of highly sialylated IgG on ADCC or CDC activity (Lin et al. 2015). Whether different methods to prepare highly sialylated IgG, the specific recombinant antibody used, or the assay systems employed to assess IgG activity underlie these inconsistent results will have to be addressed in future studies.

Of note, it was also demonstrated that different IgG subclasses were affected differentially by sialylation. Thus, mouse IgG1 antibodies showed a stronger reduction in activity compared to mouse IgG2b, further emphasizing that, especially with respect to changes of individual sugar residues, each IgG subclasses needs to be considered individually (Nimmerjahn and Ravetch 2005). A plausible explanation for these IgG subclass-specific effects is the varying baseline affinity of different IgG subclasses for different activating FcγRs. Whereas IgG1 shows a weak binding to its activating FcγR, FcγRIII, and hence may be impacted stronger by even small reductions in affinity, IgG2b has a roughly tenfold higher affinity for its major activating FcγR, FcγRIV, and thus may be more resistant to sialic acid-dependent affinity modulations (Kaneko et al. 2006). Further along these lines, IgG antibodies mediate their activity in vivo as higher order immune complexes, which may further mitigate sialylation-dependent reductions in affinity for individual FcγRs via avidity effects. Indeed, even aglycosylated IgG has some residual binding to select activating FcγRs as an immune complex (Lux et al. 2013). In addition, Scallon and colleagues observed for select antibodies that changes in antibody affinity to its

target antigen may occur upon hypersialylation, suggesting that under certain conditions not only the Fc but also the Fab fragment of the IgG molecule may be affected by sialylation (Scallon et al. 2007). In summary, more detailed further studies will be necessary to assess the impact of IgG sialylation on FcγR binding, taking into account IgG subclasses and individual FcγRs. Apart from a potential impact of highly sialylated IgG molecules to classical FcγR binding, terminal sialic acid residues were also suggested to confer an active anti-inflammatory activity to IgG (Fig. 12.3b), which will be discussed later in this review.

12.3.2 Impact of Galactose on IgG Dependent Effector Functions

With respect to IgG galactosylation, there is the long-standing observation that IgG lacking terminal galactose residues (G0 glycoform) is associated with high disease activity (Arnold et al. 2007). Apart from establishing IgG-G0 forms as a valid biomarker of active disease, it was suggested that IgG glycoforms lacking galactose residues might also have a higher pro-inflammatory activity (Fig. 12.3b) due to a better binding to activating FcγRs or the classical complement pathway. Initial evidence along these lines was provided by data demonstrating that IgG-G0 glycoforms may allow a better binding to mannose binding lectin (MBL) and hence may trigger the MBL pathway of complement activation (Malhotra et al. 1995). A later study performed in MBL deficient mice, however, demonstrated that IgG-G0 glycoforms did not lose in vivo activity and continued to operate via binding to classical FcγRs, arguing against a major contribution of the complement pathway to the activity of this IgG glycoform at least in mouse model systems (Nimmerjahn et al. 2007). In further contrast to the expectation that agalactosylated IgG glycoforms have a higher pro-inflammatory activity, more recent studies suggest that the presence of galactose residues improves IgG binding to both, FcγRs and the classical complement pathway. For example, an increased binding of galactosylated human IgG1 to FcγRIIA and FcγRIIIA was noted, which translated to an increased ADCC activity (Dashivets et al. 2015; Subedi and Barb 2016; Thomann et al. 2015); and high levels of IgG1 galactosylation also improved binding to C1q and complement-dependent cytotoxicity at least in vitro (Peschke et al. 2017). Complicating the situation even further, it was noted that highly galactosylated IgG1 molecules, present as immune complexes, are associated with an enhanced anti-inflammatory activity (Karsten et al. 2012). Thus, injection of galactosylated IgG1 immune complexes interfered with autoantibody activity in several mouse models of autoimmunity by promoting the association between FcγRIIB and Dectin-1, further suggesting that galactose residues increase IgG binding to FcγRs (Karsten et al. 2012). As discussed for terminal sialic acid residues, more studies with individual mouse and human IgG subclasses will be critical to resolve this issue. At present, however, there seems to be no link between the increased abundance of

agalactosylated IgG species in patients with infections or inflammation and a higher activity of this IgG glycosylation variant.

12.3.3 Impact of Fucose on IgG Dependent Effector Functions

The most convincing data that an altered IgG glycosylation translates into increased IgG activity exists for the penultimate fucose residue. While initial studies also pointed out that IgG1 molecules bearing bisecting *N*-acetylglucosamine residues have a higher ADCC activity (Davies et al. 2001; Umana et al. 1999), later studies demonstrated that this effect was explained due to the concomitant lack of fucose residues in these IgG preparations (Dekkers et al. 2017; Shields et al. 2002; Shinkawa et al. 2003). Non-fucosylated antibodies of all IgG subclasses bind with higher affinity to the activating FcγRIIIA, substantially increasing ADCC activity and independently of IgG subclass (Niwa et al. 2005a). Of note, antigen binding and CDC seem not to be altered by removal of core fucose (Chung et al. 2012; Niwa et al. 2005a). Therefore, generating afucosylated IgG has become a general strategy to selectively enhance therapeutic antibody binding to FcγRIIIA (Niwa et al. 2005b; Satoh et al. 2006). Mechanistically, crystal structural analysis revealed that the penultimate fucose residue of IgG comes in close contact with an N-linked sugar moiety present in FcγRIIIA, hindering a tight protein-protein interaction and thus resulting in a lower-affine interaction (Ferrara et al. 2006, 2011). It is interesting to note that this IgG glycosylation variant selectively modulates the binding to FcγRIIIA (CD16), while it does not affect IgG binding to other activating or the inhibitory FcγRIIB.

12.3.4 Impact of IgG Glycosylation on Antibody Half-Life

In general, IgG molecules exhibit quite long half-lives due to their binding to the neonatal Fc receptor (FcRn) (Roopenian and Akilesh 2007). This receptor is broadly expressed by hematopoietic and parenchymal cells and binds IgG molecules at the CH2–CH3 interface of the Fc. It protects IgG molecules from rapid degradation in lysosomes by a specific pH-dependent recycling process (Roopenian and Akilesh 2007), therefore increasing antibody half-lives. In contrast to the essential role of IgG glycosylation for binding to classical FcγRs, FcRn binding to IgG occurs in the IgG CH3 domain and thus far from the IgG-linked sugar domain. Indeed, the sugar moiety of IgG is not involved in FcRn binding as aglycosylated IgG can still bind FcRn and displays a normal half-life. Nonetheless, some IgG glycosylation variants were shown to have an altered in vivo half-life. For example, antibodies with terminal sialic acid residues were suggested to have a slightly longer half-life than

non-sialylated antibodies (Bas et al. 2019; Raju et al. 2001). Conversely, glycoproteins containing high mannose structures exhibit a reduced serum half-life due to their ability to binding to the mannose receptor (Jones et al. 2007; Kanda et al. 2007; Raju et al. 2001; Wright et al. 2000). However, another study investigating mannose enriched IgG preparations found no significant differences in half-life between complex-type and high mannose type glycans (Millward et al. 2008). Thus, while IgG glycosylation is not critical for FcRn binding, some sugar moieties may slightly impact IgG half-life in vivo.

12.3.5 IgG Sialylation and the Anti-inflammatory Activity of IgG

Since the discovery that the infusion of high doses of intravenous immunoglobulin G (IVIg) consisting of pooled serum IgG preparations from thousands of donors can suppress a wide variety of chronic inflammatory and autoimmune diseases, scientists have tried to identify the mechanism underlying this activity (Bayry et al. 2011; Schwab and Nimmerjahn 2013). As this review focuses on IgG glycosylation, we will not cover all potential molecular and cellular pathways underlying IVIg activity. The interested reader is directed to several excellent reviews covering this topic in greater detail (Bayry et al. 2011; Bussel 2006; Schwab and Nimmerjahn 2013). With respect to a potential role of IgG glycosylation in the immunomodulatory activity of IgG, the previously mentioned observation that serum IgG antibodies have reduced levels of galactosylation and sialylation during active inflammation suggested that these terminal sugar moieties might play an active role in suppressing inflammation independently of classical FcγRs. Evidence along these lines was first provided by Kaneko and colleagues in 2006, demonstrating that in animal models, IVIg lost its activity if the entire IgG sugar moiety or more selectively α2,6-linked sialic acid residues were removed (Kaneko et al. 2006). These results were further validated in a variety of other classical and humanized mouse model systems (Bozza et al. 2019; Fiebiger et al. 2015; Massoud et al. 2014; Schwab et al. 2012, 2014, 2015). Of note, several groups also noted sialic acid independent IVIg activities, suggesting that both sialic acid-dependent and -independent pathways may underly IVIg activity (Campbell et al. 2014; Issekutz et al. 2015; Leontyev et al. 2012; Othy et al. 2014). More importantly, from a clinical perspective, enriching IVIg for terminal sialic acid residues resulted in increased immunosuppressive activity, opening a new avenue for potentially enhancing IVIg activity (Anthony et al. 2008a; Kaneko et al. 2006). In an effort to replace hypersialylated IVIg infusion, soluble variants of the galactosyltransferase B4GALT1 and ST6Gal1 were generated to allow an in vivo galactosylation and sialylation of autoantibodies. Indeed, co-injection of these enzymes induced increased IgG galactosylation and sialylation at the site of inflammation, leading to an amelioration of autoimmune pathology (Pagan et al. 2018). Of note, activated platelets present at the site of autoantibody activity were

demonstrated to release sugar donor molecules required for autoantibody galactosylation and sialylation in vivo (Pagan et al. 2018).

Based on this conceptual framework, highly pure tetrasialylated IVIg preparations were generated and shown to have a superior anti-inflammatory activity in various pre-clinical model systems (Washburn et al. 2015). More recently, tetrasialylated IVIg preparations successfully passed first clinical trials in patients with Immune thrombocytopenia, suggesting that the basic concepts identified in pre-clinical mouse model systems translate to the human situation (Arroyo et al. 2019). Once approved, the data obtained in various human autoimmune disease patient cohorts will have to show how broad the clinical benefit of hypersialylated IVIg preparations is.

With respect to the molecular pathways underlying the anti-inflammatory activity of hypersialylated IgG, no unifying model has emerged yet. Starting with several studies by Anthony and colleagues, several other groups have noted that C- or I-type lectin receptors, such as DC-SIGN (murine homolog: SIGNR1), DCIR, CD22, and CD23 seem to be involved in the anti-inflammatory and immunomodulatory activity of sialylated IgG in different model systems in vivo (Anthony et al. 2008b; Fiebiger et al. 2015; Massoud et al. 2014; Pagan et al. 2018; Schwab et al. 2012; Seite et al. 2010; Wang et al. 2015). Extending these observations, it was suggested that hypersialylated IgG may directly bind to these so-called type II FcRs and induce an upregulation of FcγRIIB on innate immune effector cells via IL-33 and IL-4-producing basophils (Anthony et al. 2011; Fiebiger et al. 2015; Pincetic et al. 2014; Sondermann et al. 2013). Mechanistically, the increased expression of FcγRIIB on neutrophils and myeloid cells increases the threshold for activation via autoantibody immune complexes, thereby mitigating pro-inflammatory effector pathways and inducing resolution of inflammation. Confirming this observation, the injection of IL-4 alone was sufficient to ameliorate autoantibody-dependent inflammation via IL-4R and STAT6 signaling in myeloid cells (Anthony et al. 2011; Wermeling et al. 2013). Apart from the upregulation of FcγRIIB on innate immune effector cells, this immunomodulatory pathway was also responsible for the expansion of regulatory T cells (Treg), which play a critical role in suppressing T cell-dependent autoimmune diseases such as multiple sclerosis (Fiebiger et al. 2015). Importantly both, an upregulation of FcγRIIB and an expansion of Tregs were observed in human patients receiving IVIg therapy, validating the results obtained in mouse model systems (Ephrem et al. 2008; Schwab et al. 2015; Tackenberg et al. 2009). In addition to acting on innate immune cells, sialylated IgG molecules were shown to feedback on B cells via CD23. Binding to CD23 induced an upregulation of the inhibitory FcγRIIB on germinal center B cells, leading to a higher threshold for B cell activation and generation of higher affine antibody responses (Wang et al. 2015). With respect to autoimmune diseases, this pathway may block the continuous production of autoantibodies by newly developing autoreactive B cells in the germinal center and via induction of apoptosis in autoantibody-producing plasma cells, and hence explain some of the long-lasting effects of IVIg therapy.

However, several publications challenged the concept that IgG sialylation allows a direct binding of IgG to type II FcRs via altering IgG structure (Temming et al.

2019; Yu et al. 2013). Thus, these receptors may still be involved in the immunomodulatory pathway of IVIg activity but not represent the initial triggering receptor setting the whole anti-inflammatory cascade into motion. As different C-type lectin receptors seemed to be involved depending on the organ affected by the disease, a better understanding of local cell types and receptors participating in IVIg activity would be very helpful. In summary, more studies will be necessary to resolve the exact pathways underlying the sialic acid-dependent anti-inflammatory activities of IVIg in different disease entities. The promising first results of hypersialylated IVIg in human clinical trials may help to resolve these issues in the near future by allowing to study immunological effects triggered via hypersialylated IVIg preparations in human patient populations.

12.4 Impact of Glycosylation on IgA Function

Unlike the well-established connection between IgG glycosylation and modulation of IgG effector function, the impact of IgA glycosylation on IgA effector functions is less well understood (reviewed in (Arnold et al. 2005, 2007); schematic representation see Fig. 12.1). This is partly due to the fact that mice and humans significantly differ with respect to this antibody isotype. Thus, mice only have one IgA subclass and lack an orthologue to the human FcαRI (CD89). IgA is the most abundant immunoglobulin on inner body surfaces and plays an essential role in mucosal homeostasis of the gastrointestinal, respiratory, and genitourinary tract (Kerr 1990). Besides its transport to mucosal tissues by plasma cells residing in the lamina propria, it is also the second most abundant immunoglobulin in the serum (Hansen et al. 2019; Kerr 1990). Secretory IgA and serum IgA differ with respect to secretory IgA being present as a dimer in a complex with the joining chain and the secretory component, and serum IgA being present predominantly in a monomeric form (Fig. 12.1) (Kerr 1990). The two IgA subclasses, IgA1 and IgA2, can be further subdivided into two IgA2 allotypic variants, named IgA2m(1) and IgA2m(2) (Woof and Kerr 2006). IgA1 and IgA2 differ by 13 amino acids found in the hinge region of IgA1, which is highly O-glycosylated in IgA1 molecules (van Egmond et al. 2001). IgA1 predominates in the serum, whereas IgA1 and IgA2 are more evenly distributed on mucosal surfaces (van Egmond et al. 2001). Apart from simple pathogen neutralization, more recent studies suggest that IgA may also actively participate in the regulation of immune responses (Hansen et al. 2019). The major effector cells expressing CD89 are monocytes, macrophages, neutrophils, eosinophils, and monocyte-derived dendritic cells, which also express FcγRs. In further similarity to activating FcγRs, CD89 also associates with the common FcRγ-chain for signal transduction (Hansen et al. 2019; van Egmond et al. 2001) and is also characterized by a low affinity (K_a approximately 10^6 M^{-1}), resulting in a rapid dissociation of monomeric IgA (Wines et al. 1999). Binding of IgA as an immune complex can trigger a variety of effector responses, like the release of cytokines and chemokines, degranulation, phagocytosis, or ADCC (Bakema and van Egmond 2011). Of note,

several studies have shown that IgA may contribute to the pathology of autoimmune diseases, like rheumatoid arthritis, celiac disease or inflammatory bowel disease, suggesting that IgA can be a driver of inflammation (reviewed in (Hansen et al. 2019)). In contrast, others have noted that serum IgA may exert anti-inflammatory effects and protect against autoimmune responses (Lecocq et al. 2013; Pilette et al. 2010; Rossato et al. 2015). Thus, in line with IgG, IgA molecules may have a dual functionality promoting both, pro- and anti-inflammatory immune responses, raising the question to what extent IgA glycosylation may impact these different effector functions.

In contrast to the single sugar moiety in the IgG Fc domain, which is largely buried in the hydrophobic pocket between the two IgG heavy chains, the different sugar moieties of IgA are exposed on the protein surface. Compared to IgG, glycosylation of IgA is more complex and differs between IgA1 and IgA2 subclasses. While IgA1 molecules contain two conserved N-glycosylation sites (N144 in the Cα2 and N340 in the Cα3 domain) and several sites of O-glycosylation in the extended hinge region of each IgA heavy chain, IgA2 has four conserved N- (N47 in the Cα1, N131 and N205 in the Cα2, and N327 in the Cα3 domain) but no O-glycosylation sites (Fig. 12.1) (de Haan et al. 2020). Whereas O-glycans consist of one core *N*-acetylgalactosamine with β1,3-linked galactose residues, which can also be sialylated (Fig. 12.1) (Deshpande et al. 2010; Franc et al. 2013; Novak et al. 2013), the majority of N-glycosylation sites of IgA are of the complex-type and vary between IgA1 and IgA2. Creating further complexity, IgA glycosylation in the serum differs from glycosylation of secretory IgA (Plomp et al. 2018; Steffen et al. 2020). For example, almost 90 % of serum IgA is either mono- or di-sialylated while secretory IgA has only 13 % sialylated glycan structures (Arnold et al. 2007; Plomp et al. 2018). Furthermore, secretory IgA is dominated by glycan structures terminating in galactose (23%) and *N*-acetylglucosamine (52%) and contains 75% bisecting *N*-acetylglucosamine and 50% core fucose residues. With 29% bisecting *N*-acetylglucosamine and 32% fucose residues, these branching sugar residues are present at a much lower abundance in serum IgA1. Moreover, secretory IgA dimers are covalently linked with the joining chain and the secretory component, which also carries complex N-glycosylation sites (Arnold et al. 2007; Deshpande et al. 2010). Although IgA molecules have no C1q binding site, it was shown that secretory IgA contains exposed mannose and *N*-acetylglucosamine residues, which are masked by the secretory component but can be unmasked upon interaction with bacteria allowing complement activation via mannose-binding lectin, promoting pathogen opsonization and phagocytosis (Royle et al. 2003). More recent publications revealed that the composition of individual N-linked sugar moieties within IgA might differ (Chandler et al. 2019; Steffen et al. 2020). For example, the N144 and N131 associated sugar moieties in IgA1 and IgA2, respectively, were virtually non-fucosylated, whereas all other N-linked sugar structures carried fucose residues. While all N-linked sugar structures in both, IgA1 and IgA2 were highly sialylated, the N340 (IgA1) and N327 (IgA2) sugar moieties showed mostly di-sialylated sugar residues, whereas the predominant glycoform in all other N-linked sugar structures was mono-sialylated. Of note, a slightly higher level of di-sialylated sugar structures

was noted on IgA1 molecules, which had a lower capacity to activate neutrophils and cause pro-inflammatory effector functions (Steffen et al. 2020). Interestingly, the global removal of sialic acid residues seemed to specifically enhance the pro-inflammatory activity of IgA1, suggesting that sialic acid residues specifically regulated IgA1 activity (Steffen et al. 2020). In line with a model in which IgA sialylation regulates binding to CD89, Basset and colleagues noted an enhanced binding of neuraminidase treated and reduced binding of hypersialylated IgA to CD89 expressing cells (Basset et al. 1999). In stark contrast to IgG, a similar effect was noted for PNGaseF treated IgA1, in which all of the N-linked sugar domains are removed, indicating that in general N-linked sugar domains are not essential for or may even limit IgA binding to CD89 (Gomes et al. 2008; Mattu et al. 1998; Oortwijn et al. 2007; Steffen et al. 2020). With respect to IgA half-life, it is important to note that not FcRn but rather the hepatic asialo receptor is involved in IgA removal from the serum. As this receptor specifically recognizes asialylated proteins, this may lead to different half-lives of sialylated (longer half-life) compared to non-sialylated (shorter half-life) IgA molecules in vivo. Combined with the generally low serum half-life of IgA this creates a complex scenario when trying to distinguish the effects of sialylation on IgA half-life and CD89 dependent effector functions. Although much more work is necessary to fully understand the impact of the individual sugar moieties on IgA function, the most recent evidence suggests that similar to IgG, sialylation may modulate IgA effector functions.

12.5 Impact of Glycosylation on IgE Function

IgE is the least abundant immunoglobulin in the serum and is critical for defending the host against infections with parasites. In addition, the pathogenic role of IgE as a trigger of allergic responses is a major clinical problem, necessitating an in-depth understanding of IgE function (Gould and Sutton 2008; Gounni et al. 1994). In the body, IgE is bound to its high-affinity receptor, FcεRI (K_a approximately 10^{10} M^{-10} (Young et al. 1995)) on mast cells and basophils, where it triggers the release of inflammatory mediators, like histamine, tumor necrosis factor (TNF), and prostaglandin D2 upon antigen recognition (Galli and Tsai 2012; Gould and Sutton 2008). Furthermore, CD23 (also known as FcεRII) has been identified as low-affinity receptor for IgE, which is expressed on T and B cells, follicular dendritic cells, and epithelial cells (Armitage et al. 1989; Rieber et al. 1993; Yu et al. 2003). CD23 can occur either in a soluble or in a membrane-bound form and is important for the regulation of IgE homeostasis (Shade et al. 2019) and the elimination of intracellular pathogens (Vouldoukis et al. 1995). IgE molecules consist of four Cε heavy chain domains with a rigid hinge region (Arnold et al. 2005; Oettgen 2016) and are the most heavily glycosylated immunoglobulins (Dorrington and Bennich 1978) (Fig. 12.1). It has seven N-glycosylation sites in the IgE heavy chain, with N383 being non-glycosylated, N394 carrying only oligomannose structures, and the remaining sites being occupied by complex glycans (Baenziger et al. 1974a, b;

Dorrington and Bennich 1978; Shade et al. 2020). Although it has been described that glycan removal leads to reduced FcεRI binding (Bjorklund et al. 1999; Nettleton and Kochan 1995), others found almost no impact of IgE glycosylation on its receptor binding capacity (Basu et al. 1993; Vercelli et al. 1989). Moreover, several early studies tried to analyze the complex glycan structure of IgE (Baenziger et al. 1974a, b; Dorrington and Bennich 1978; Nettleton and Kochan 1995). However, as the level of IgE is very low those studies were usually performed on myeloma-derived IgE or did not distinguish between individual N-glycosylation sites making it difficult to draw conclusions about site-specific glycosylation patterns within IgE. The first insights into site-specific IgE glycosylation was provided by Plomp and colleagues, who also compared serum IgE glycosylation between myeloma patients, healthy and hyperimmune donors (Plomp et al. 2014). This study demonstrated that IgE from myeloma patients was characterized by decreased levels of bisecting *N*-acetylglucosamines, which was further associated with tumor progression (Balog et al. 2012; Miwa et al. 2012). More recently, a detailed site-specific IgE glycan analysis demonstrated that the N-linked sites at N140, N168, N218, and N265 (N21, N49, N99 and N146 according to UniProt numbering) are almost completely mono- or di-sialylated while only 30% of the N371 (N252 according to UniProt numbering) were glycosylated; no sugar moiety was present at the N383 (N264 according to UniProt numbering) site, consistent with previous results (Arnold et al. 2004; Baenziger et al. 1974a, b; Dorrington and Bennich 1978; Plomp et al. 2014; Shade et al. 2020; Wu et al. 2016). With respect to the impact of the different N-linked glycosylation sites on binding to the high-affinity FcεRI, several studies suggest a prominent role for the N394-linked (N275 according to UniProt numbering) high mannose structure, which is conserved in IgE molecules of all mammalian species (Bjorklund et al. 1999; Shade et al. 2019). A selective enzymatic removal of the N394 sugar moiety altered the secondary structure of IgE prevented binding to FcεRI on mast cells and attenuated IgE-mediated anaphylaxis in vivo (Shade et al. 2015). In addition, some minor effects of the other N-linked complex sugar structures have been identified (Shade et al. 2019). While removal of the complex glycans at position N265 and/or N371 minimally affected FcεRI binding, mutation of the N-linked sites in the Fab region showed slightly reduced IgE-mediated mast cell degranulation, suggesting a possible interaction of IgE Fab glycans with its antigen (Shade et al. 2015, 2019). Moreover, galectins, a class of proteins that bind specifically to β-galactoside sugars and are involved in many physiological functions such as inflammation, immune responses, cell migration, autophagy, and signaling (Johannes et al. 2018), were shown to bind IgE molecules, suggesting that complex IgE glycans might be involved in IgE effector functions (Shade et al. 2019). High-affinity binding of IgE glycans to galectins can regulate IgE activity and have an anti-allergic effect by preventing IgE-antigen complex formation. By comparing IgE glycosylation between atopic and non-atopic individuals, reduced amounts of bisecting *N*-acetylglucosamines and terminal galactose residues but increased levels of sialic acids were detected in allergen-specific IgE. Of note, removal of sialic acid residues attenuated IgE-dependent effector cell degranulation and anaphylaxis (Shade et al. 2020).

Thus, in contrast to IgG and IgA isotypes, where terminal sialic acid residues conferred a reduced pro-inflammatory activity, terminal sialic acid residues were critical for IgE-dependent effector functions. This finding would be in line with the Th2 cytokine-dependent immune responses triggered by parasite infections, which may change the B cell-intrinsic glycosylation machinery towards a high sialylation state.

12.6 Summary and Outlook

In summary, research over the last decade has firmly established that glycosylation plays an active role in modulating the activity of different antibody isotypes in vivo. While the multiple and complex sugar moieties present in IgA and IgE create a way more complex scenario compared to IgG, several studies have started to show that either select sugar moieties or individual sugar residues such as sialic acids seem to play a crucial role in fine-tuning antibody activity. With respect to the pathways determining how a plasma cell glycosylates different antibody isotypes, much more studies are necessary to obtain a clear picture of the underlying factors involved in modulating antibody glycosylation in vivo. The observation that certain cytokines or organ environments in which the plasma cell resides may impact antibody glycosylation provide a first roadmap for further investigations. Finally, the encouraging data from the clinical use of glycoengineered antibodies underscores the translational impact this field of research already has and surely will have in the near future.

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Compliance with Ethical Standards

Conflict of Interest Falk Nimmerjahn declares that he has no conflict of interest. Anja Werner declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 13
Immunoglobulin G Glycosylation in Diseases

Marija Pezer

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Abstract Changes in immunoglobulin G (IgG) glycosylation pattern have been observed in a vast array of auto- and alloimmune, infectious, cardiometabolic, malignant, and other diseases. This chapter contains an updated catalog of over 140 studies within which IgG glycosylation analysis was performed in a disease setting. Since the composition of IgG glycans is known to modulate its effector functions, it is suggested that a changed IgG glycosylation pattern in patients might be involved in disease development and progression, representing a predisposition and/or a functional effector in disease pathology. In contrast to the glycopattern of bulk serum IgG, which likely relates to the systemic inflammatory background, the glycosylation profile of antigen-specific IgG probably plays a direct role in disease pathology in several infectious and allo- and autoimmune antibody-dependent diseases. Depending on the specifics of any given disease, IgG glycosylation read-out might therefore in the future be developed into a useful clinical biomarker or a supplementary to currently used biomarkers.

Keywords IgG glycosylation · Differential glycosylation · Disease · Biomarker

M. Pezer (✉)
Glycoscience Research Laboratory, Genos Ltd., Zagreb, Croatia
e-mail: mpezer@genos.hr

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Abbreviations

ACPA	Anti-citrullinated protein antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Asn	Asparagine
CH2	Constant heavy 2
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcγRs	Fcγ receptors
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
GlcNac	*N*-acetylglucosamine
HFD	High-fat diet
HDFN	Haemolytic disease of the fetus and newborn
IgG	Immunoglobulin G
IVIg	Intravenous immunoglobulin
RA	Rheumatoid arthritis

13.1 Introduction

Since the first reports on glycans attached to immunoglobulin G (IgG) in the 1970s (Ciccimarra et al. 1976; Williams et al. 1973; Koide et al. 1977; Hymes et al. 1979) and the seminal papers by Parekh and al. on the association of a changed IgG glycome composition with a diseased status and aging, (Parekh et al. 1985, 1988) IgG glycans are today universally recognized as modulators of IgG activity (Yamaguchi and Barb 2020). The importance of IgG glycome composition is implied in various physiological and pathological states. IgG glycans are discussed as potential contributors to disease development and progression, as well as a diagnostic, prognostic, and follow-up biomarker. This chapter is a brief update and extension of our comprehensive review on IgG glycosylation in aging and diseases published 3 years ago (Gudelj et al. 2018a), with a focus on the potential functionality of the skewed IgG glycosylation pattern. The table presents the updated list of publications that examined IgG glycosylation in various diseased states.

13.2 IgG Glycans are an Integral Structural and Functional Part of the Molecule

IgG glycans represent about 15% of the molecule's weight (Arnold et al. 2007). Each IgG molecule contains an N-glycan covalently attached to the conserved asparagine (Asn) at position 297 within the Fc region on each of the two heavy chains (Shade and Anthony 2013). In addition, 15–20% IgG molecules contain an

N-glycan within the Fab region, attached to the asparagine within an N-glycosylation sequon formed by somatic hypermutation during affinity maturation (Dunn-Walters et al. 2000; van de Bovenkamp et al. 2016).

Fc N-glycans are placed in the cavity between the CH2 domains of the two opposing heavy chains (Pincetic et al. 2014; Deisenhofer et al. 1976) and are important for the molecule's structural integrity, stability, and serum-half life (Boune et al. 2020; Cymer et al. 2018). They are also involved in the modulation of IgG effector functions, by affecting the molecule's affinity toward its ligands and receptors: type I and type II Fc receptors, C1q complement component, mannan-binding lectin, etc. (Pincetic et al. 2014; Peschke et al. 2017; Malhotra et al. 1995; Dekkers et al. 2017). Although markedly less explored than Fc glycans, Fab glycans are also reported to affect IgG's biological properties and effector functions, such as half-life, stability, solubility, and antigen-binding (van de Bovenkamp et al. 2016, 2018a; Wu et al. 2010; Wright et al. 1991; Higel et al. 2016; Liu 2015, 2018).

13.3 IgG Glycans Affect IgG Functions

The composition of both Fab and Fc glycans has been confirmed to influence IgG functionality and activity. Since this has been described in detail in Chap. 12, the main findings are only briefly summarized here as a reminder for the reader.

13.3.1 Fc Glycans

Due to the positioning of the Fc N-glycan at the Asn-297, structural differences of the N-glycans attached to the Fc region influence the affinity to the IgG ligands and receptors that interact with IgG at the CH2 domain and the CH2-CH3 domain interface (Dekkers et al. 2017; Reusch and Tejada 2015; Li et al. 2017; Wada et al. 2019; Vidarsson et al. 2014).

Core-Fucosylation Contrary to most other plasma proteins, over 90% of all Fc glycans are core-fucosylated (fucosylated glycans, F) (van de Bovenkamp et al. 2016; Štambuk et al. 2020; Baković et al. 2013; Clerc et al. 2016). The lack of core fucose significantly increases the IgG's affinity for the Fcγ receptor III (FcγRIII), both A and B, enhancing the FcγRIII-mediated effector functions, particularly the antibody-dependent cell-mediated cytotoxicity (ADCC) (Dekkers et al. 2017; Shields et al. 2002; Shinkawa et al. 2003). This prominent effect of alternative Fc glycosylation on the IgG function found its application in the industrial production of therapeutic monoclonal antibodies (Garber 2018).

Bisection Up to 10% of all IgG Fc glycans are bisected, i.e., contain a bisecting *N*-acetylglucosamine (GlcNAc) (bisected glycans, B) (van de Bovenkamp et al. 2016). Since the presence of GlcNAc and core fucose, to a degree, preclude each

other during glycan synthesis (Benedetti et al. 2017; Schuster et al. 2005; Ferrara et al. 2006), the increase in binding affinity fo FcγRIII sometimes associated with bisected glycans (Umaña et al. 1999; Davies et al. 2001; Lifely et al. 1995) cannot be easily uncoupled from the same effect observed for core-fucosylated IgG glycans (Shinkawa et al. 2003).

Galactosylation Galactosylation is the IgG glycosylation trait with the most pronounced inter-individual variation (Huhn et al. 2009; Gornik et al. 2012). On average, about 35% of IgG Fc glycans contain no terminal galactose residues (agalactosylated glycans, G0), about 35% contain one (monogalactosylated glycans, G1), and about 15% contain two terminal galactoses (digalactosylated glycans, G2) (Baković et al. 2013; Huffman et al. 2014). Terminal galactoses modulate IgG inflammatory potential by affecting binding affinities to complement components and FcγRs. Agalactosylated Fc glycans are considered to act pro-inflammatory by activating the complement through the alternative pathway (Banda et al. 2008), and the lectin pathway after binding to the mannose-binding lectin (Malhotra et al. 1995; Ji et al. 2002; Arnold et al. 2006). Galactosylation was also held responsible for the anti-inflammatory activity of immune complexes by binding to the inhibitory FcγRIIB (Karsten et al. 2012). However, Fc galactosylation has also been reported to enhance complement-dependent cytotoxicity (CDC) through the classical complement pathway by increasing the IgG's affinity for the C1q complement component (Peschke et al. 2017; Boyd et al. 1995; Hodoniczky et al. 2005). Likewise, by increasing the affinity of IgG for FcγRs, it enhances the downstream processes mediated by FcγRs, in particular ADCC (Dekkers et al. 2017; Kumpel et al. 1994, 1995; Houde et al. 2010; Subedi and Barb 2016). We should therefore not rush to proclaim terminal IgG galactosylation simply "anti-inflammatory," before considering the entire context and the nature and extent of IgG involvement in the process we are investigating.

Sialylation On average, 10–15% of IgG Fc glycans carry a single terminal sialic acid (monosialylated glycans, S1) or two sialic acids (disialylated glycans, S2) (Baković et al. 2013; Huffman et al. 2014). Similar to terminal galactosylation, sialylation is most often discussed as a modulator of IgG functions regarding inflammation (Böhm et al. 2014).

The importance of sialylation became evident when the presence of the sialylated Fc fraction was discovered indispensable for the anti-inflammatory activity of the intravenous immunoglobulin (IVIg) preparation in a K/BxN serum-transfer mouse model of RA (Kaneko et al. 2006). Mouse studies on several antibody-dependent autoimmune disease models helped elucidate the mechanistic pathway for its activity, starting with the binding of the sialylated Fc fraction to specific ICAM-3 grabbing non-integrin-related 1 (SIGN-R1) on the surface of splenic macrophages and ending in enhanced FcγRIIB expression on the effector macrophages (Kaneko et al. 2006; Schwab and Nimmerjahn 2013; Anthony et al. 2008, 2011; Schwab et al. 2012, 2014; Washburn et al. 2015; Galeotti et al. 2017; Fiebiger et al. 2015). However, this finding did not hold in several other in vitro and in vivo models, nor human studies (Galeotti et al. 2017; Guhr et al. 2011; Leontyev et al. 2012; Campbell et al. 2014; Temming et al. 2019). This confirms the well-established

notion that the IVIg mode of action is complex and tightly connected with the corresponding immune context.

Depending on the sialylation status of the Fc glycan, the Fc domain is suggested to adopt either an "open" or a "closed" conformation, for sialylated and asialylated glycans, respectively. The "open" conformation favors binding to the type I FcγRs, whereas the "closed" conformation favors the binding of type II FcRs (Pincetic et al. 2014; Sondermann et al. 2013). Terminal sialylation is thus proposed to act as a switch between two distinct immunological effector functions.

To summarize—agalactosylated, asialylated, and bisected IgG molecules are often simply described as "pro-inflammatory," and terminally galactosylated and sialylated IgG molecules as "anti-inflammatory," while afucosylated IgG has an augmented capacity to trigger ADCC through enhanced FcγRIIIA binding. We should, however, always bear in mind that this generalization is a simplification, and exercise caution when considering its implications.

13.3.2 Fab Glycans

As expected, Fab glycans are mostly reported to affect antigen-binding (Wright et al. 1991; Coloma et al. 1999; Schneider et al. 2015; Wallick et al. 1988; Tachibana et al. 1997; Leibiger et al. 1999; Khurana et al. 1997; Man Sung Co et al. 1993; Fujimura et al. 2000; Van De Bovenkamp et al. 2018b). Besides the obvious, they are also suggested to influence IgG aggregation and precipitation (Courtois et al. 2016), immune complex formation (Gutierrez et al. 2006), and have a role in the IVIg mode of action (Käsermann et al. 2012; Wiedeman et al. 2013; Massoud et al. 2014; Séïté et al. 2010, 2014).

13.4 Regulation of IgG Glycosylation

IgG glycosylation is a complex trait, influenced by both, genetics (Menni et al. 2013; Pučić et al. 2011; Klarić et al. 2020) and the environment (Štambuk et al. 2020; Yu et al. 2016; Krištić et al. 2014; De Jong et al. 2016). More precisely, the compound IgG glycosylation pattern seems to be, to different degrees, modulated by IgG aminoacid sequence (Lund et al. 1996; Zaitseva et al. 2018; Yu et al. 2013), the intra- and extracellular milieu affecting the glycosylation machinery (Ohtsubo and Marth 2006; Oefner et al. 2012; Bartsch et al. 2020; Hess et al. 2013; Canellada et al. 2002; Gutiérrez et al. 2001; Miranda et al. 2005; Wang et al. 2011; Pfeifle et al. 2017; Liu et al. 2014; Fan et al. 2015), and environmental factors (Novokmet et al. 2014; Greto et al. 2020; Ercan et al. 2017; Engdahl et al. 2018; Klasić et al. 2018; Tijardović et al. 2019; Sarin et al. 2019; Peng et al. 2019). Solving the outstanding question of IgG glycosylation regulation would likely bring us one step closer to understanding the possible functionality of changes in IgG glycan composition in different physiological and pathological states.

13.5 Common IgG Glycosylation Pattern in Inflammatory Diseases and Aging

Advances in the development of high-throughput glycomic and glycoproteomic analyses (Huhn et al. 2009; Mariño et al. 2010; Trbojević-Akmačić et al. 2016, 2017) have enabled a significant number of large-scale epidemiological studies examining total IgG glycosylation pattern in diseased *vs.* healthy control subjects (Štambuk et al. 2020; Singh et al. 2020; Lemmers et al. 2017; Menni et al. 2018; Šimurina et al. 2018; Theodoratou et al. 2016; Wahl et al. 2018). In many of the diseases that were studied, a similar pattern emerged: diseased subjects often exhibited a decreased abundance of galactosylated, sialylated, and—occasionally—an increased abundance of bisected bulk IgG glycans when compared to healthy controls (Fig. 13.1). In addition, the trend was often associated with disease severity and reverted to baseline values upon successful application of therapy. Interestingly, the same pattern that was observed in diseases with an inflammatory component was also evident in aging subjects (Fig. 13.1) (Gudelj et al. 2018a; Lauc 2016). This "pro-inflammatory IgG glycome composition" is likely associated with

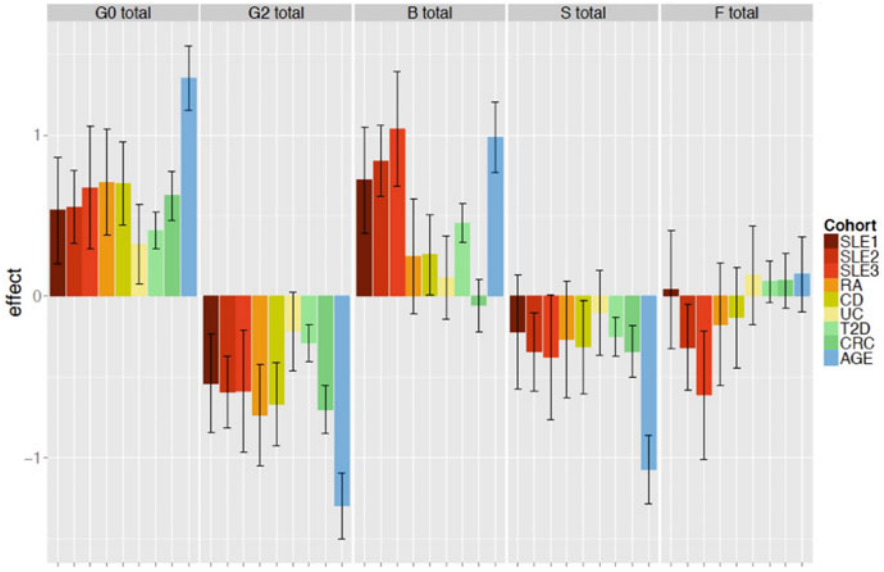

Fig. 13.1 General patterns of IgG glycosylation changes are similar in several diseases and aging. The effect (shown on the *y*-axis) is shown as the difference between means of case and control populations (for aging, population over *vs.* population under 50 years of age), expressed in standard deviations. The whiskers represent the 95% confidence interval. The reference populations for disease cohorts are age- and sex-matched healthy controls. *SLE* systemic lupus erythematosus, *RA* rheumatoid arthritis, *CD* Crohn's disease, *UC* ulcerous colitis, *T2D* type 2 diabetes mellitus, *CRC* colorectal carcinoma. *G0* agalactosylated glycans, *G2* digalactosylated glycans, *B* bisected glycans, *S* sialylated glycans, *F* core-fucosylated glycans. Reused with permission from Lauc (2016)

the common background inflammatory component of the studied diseases. In some cases, it might reflect a predisposition toward disease development, or/and even be involved as an effector of inflammation. Additionally, it might represent a consequence of environmental exposure to antigens through a lifetime or unhealthy lifestyle choices.

Indeed, the composition of IgG glycome was reported to associate with many physiological and biochemical traits, as well as with traits correlated to inflammation and poor metabolic health (Gudelj et al. 2018a) and the expected lifespan (Štambuk et al. 2020). IgG glycome was thus suggested to be a biomarker of general immune activation (De Jong et al. 2016), while we propose total IgG glycoprofile can be positioned as a read-out of a general state of health, i.e. biological age (Vilaj et al. 2019).

13.6 Role of Skewed IgG Glycosylation in Diseases

When we take into account the complexity of the IgG's multiple roles in our immune system, it is no wonder there is no single common interpretation of the altered IgG glycopattern across the wide spectrum of diseases (Table 13.1). The multiple pleiotropic loci, i.e. shared associations of IgG glycome composition and autoimmune, inflammatory, and other diseases (Klarić et al. 2020; Lauc et al. 2013), as well as the changes in IgG glycopattern preceding disease development—such as in the case of RA (Gudelj et al. 2018b) and cardiovascular diseases (Menni et al. 2018)—suggest that a skewed bulk serum IgG glycoprofile might reflect a disease risk or predisposition. This predisposition can manifest through an inherited (Klarić et al. 2020; Lauc et al. 2013) or acquired propensity for inflammation modulation (Franceschi et al. 2018).

In most other cases, when it comes to glycosylation of the bulk serum IgG, the role of a shifted glycosylation pattern is not clear. As already mentioned, decreased galactosylation and sialylation often accompany diseases that involve an inflammatory immune response. The evidence that would enable us to unambiguously determine whether the "pro-inflammatory" IgG glycoforms represent one of many drivers of disease pathology or merely byproducts of the inflamed immune system is still lacking. The current consensus is that total IgG glycopattern is likely relevant in the general modulation of the immune activation threshold.

In some cases, however, a clear link/evidence for the functionality of IgG glycans is provided. A mouse study investigating the link between obesity and the development of hypertension resulted in a very intriguing observation. Hyposialylated IgG from mice in which obesity was induced by a high-fat diet (HFD) induced an elevated blood pressure when transferred to IgG-deficient mice. Moreover, supplementing HFD-feed mice with a sialic acid precursor, *N*-acetyl-D-mannosamine (ManNAc), resulted in the restoration of the baseline level of IgG sialylation and protected them from obesity-induced hypertension development (Peng et al. 2019). This finding thus demonstrated the functional role of IgG glycans

Table 13.1 Diseases exhibiting an altered serum IgG glycosylation profile

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G	**Inflammatory diseases and states** Takayasu's arteritis (Hernandez-Pando et al. 1994) Adult periodontal disease (Novak et al. 2005) Nonalcoholic steatohepatitis (Vanderschaeghe et al. 2018) IgG4-related disease (Culver et al. 2019) Primary sclerosing cholangitis (Culver et al. 2019) **Autoimmune diseases** Rheumatoid arthritis—total (Parekh et al. 1985, 1988; Bond et al. 1996; Van de Geijn et al. 2009; Young et al. 1991; Axford et al. 1992; Engdahl et al. 2018; Gudelj et al. 2018b; Gindzienska-Sieskiewicz et al. 2007; Tomana et al. 1988; Bodman-Smith et al. 1996; Gińdzieńska-Sieśkiewicz et al. 2016; Pekelharing et al. 1988; Pilkington et al. 1995), ACPA (Ercan et al. 2010; Rombouts et al. 2015; Bond et al. 1997, 2018), RF (Matsumoto et al. 2000) Osteoarthritis (Parekh et al. 1985; Bond et al. 1997) Juvenile onset rheumatoid arthritis (Parekh et al. 1988; Flögel et al. 1998; Sumar et al. 1991; Ercan et al. 2012; Cheng et al. 2017) Systemic lupus erythematosus (Tomana et al. 1988, 1992; Pilkington et al. 1995; Bond et al. 1997; Vučković et al. 2015) Lupus nephritis (Bhargava et al. 2021) Inflammatory bowel disease: Crohn's disease and ulcerative colitis (Šimurina et al. 2018; Tomana et al. 1988; Bond et al. 1997; Dubé et al. 1990; Go et al. 1994; Shinzaki et al. 2008; Nakajima et al. 2011; Trbojevic Akmacic et al. 2015; Parekh et al. 1989; Miyoshi et al. 2016) Sjögren's syndrome (Bond et al. 1996, 1997) Neonatal lupus (Pilkington et al. 1996a) Spondyloarthropathy (Bond et al. 1997; Leirisalo-Repo et al. 1999) ANCA-associated vasculitis—total (Holland et al. 2002, 2006; Espy et al. 2011; Kemna et al. 2017; Wuhrer et al. 2015), ANCA (Kemna et al. 2017; Wuhrer et al. 2015) Coeliac disease (Cremata et al. 2003) Lambert–Eaton myasthenic syndrome (Selman et al. 2011) Myasthenia gravis (Selman et al. 2011) Myositis (Perdivara et al. 2011) Guillain–Barré syndrome (Fokkink et al. 2014a; b)	**Alloimmune diseases** Fetal or neonatal alloimmune thrombocytopenia—anti-HPA (Sonneveld et al. 2016; Wuhrer et al. 2009) Hemolytic disease of the fetus and newborn—anti-c, anti-E (Sonneveld et al. 2017a) **Cancers** Thyroid cancer (Chen et al. 2012) Multiple myeloma (Mittermayr et al. 2017) Mammary gland hyperplasia (Meng et al. 2020) **Infectious diseases** Measles—anti-measles (Larsen et al. 2020) Mumps—anti-mumps (Larsen et al. 2020) Parvovirus-B19 infection—anti-B19 (Larsen et al. 2020) COVID-19—anti-S (Larsen et al. 2020), anti-N (Larsen et al. 2020) RSV infection—anti-RSV (van Erp et al. 2020) Tuberculosis—antigen-specific (Lu et al. 2020) **Other diseases** Parkinson's disease (Russell et al. 2017)

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Table 13.1 (continued)

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	Poor glycemic control and impaired renal function in type I diabetes (Bermingham et al. 2018) Autoimmune hemolytic anemia—total and anti-RBC (Sonneveld et al. 2017b) Membranous nephropathy (Haddad et al. 2021) **Alloimmune diseases** Hemolytic disease of the fetus and newborn—anti-K (Sonneveld et al. 2018) **Cancers** Multiple myeloma (Nishiura et al. 1990; Aurer et al. 2007) Bone disease in multiple myeloma (Westhrin et al. 2020) Ovarian cancer—total (Gerçel-Taylor et al. 2001; Saldova et al. 2007; Alley et al. 2012; Qian et al. 2013; Ruhaak et al. 2016), tumor-reactive (Gerçel-Taylor et al. 2001) Prostate cancer (Kanoh et al. 2004a, 2008, 2009; Kazuno et al. 2016) Non-small cell cancer (Kanoh et al. 2006) Gastric cancer (Kanoh et al. 2004b, 2008; Bones et al. 2010, 2011; Kodar et al. 2012) Lung cancer (Kanoh et al. 2004b, 2008; Chen et al. 2013) Colorectal carcinoma (Theodoratou et al. 2016; Vučković et al. 2016) Breast cancer (Kawaguchi-Sakita et al. 2016) Malignant hematological diseases[a] (de Haan et al. 2018a) **Infectious diseases** Leprosy—Erythema nodosum leprosum (Filley et al. 1989) Tuberculosis (Rook et al. 1994; Pilkington et al. 1995, 1996b; Parekh et al. 1989; Filley et al. 1989; Rademacher et al. 1988; Lu et al. 2016) Infective endocarditis (Bond et al. 1997) HIV infection—total (Ackerman et al. 2013; Moore et al. 2005; Muenchhoff et al. 2020), anti-HIV (Ackerman et al. 2013; Larsen et al. 2020) Hepatitis C: liver fibrosis, cirrhosis—anti-Gal (Mehta et al. 2008) Hepatitis B: chronic infection (Ho et al. 2015); liver cirrhosis – total (Ho et al. 2015), anti-Gal (Mehta et al. 2008) Visceral leishmaniasis (Gardinassi et al. 2014) CMV infection—anti-CMV (Larsen et al. 2020)	

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Table 13.1 (continued)

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	COVID-19 [139] **Other diseases** Castleman's disease (Nakao et al. 1991) Galactosaemia (Coss et al. 2012; Knerr et al. 2015; Maratha et al. 2016; Stockmann et al. 2016; Coman et al. 2010; Coss et al. 2014) Alzheimer's disease (Lundström et al. 2014) Asthma? (De Jong et al. 2016; Pezer et al. 2016) Chronic kidney disease (Barrios et al. 2016) Hypertension (Wang et al. 2016; Gao et al. 2017) Type II diabetes (Lemmers et al. 2017; Li et al. 2019) Nonalcoholic fatty liver disease (Zhao et al. 2018) Ischemic stroke (Liu et al. 2018) Hyperuricemia (Hou et al. 2019) Diabetic retinopathy (Wu et al. 2021)	
S	**Inflammatory diseases and conditions** Primary sclerosing cholangitis (Culver et al. 2019) **Autoimmune diseases** Rheumatoid arthritis—total (Parekh et al. 1985; Engdahl et al. 2018; Gudelj et al. 2018b; Gińdzieńska-Sieśkiewicz et al. 2016), ACPA (Scherer et al. 2010), RF (Matsumoto et al. 2000) Osteoarthritis (Parekh et al. 1985) ANCA-associated vasculitis—total (Espy et al. 2011; Kemna et al. 2017; Wuhrer et al. 2015), ANCA (Kemna et al. 2017; Wuhrer et al. 2015) Systemic lupus erythematosus—total (Vučković et al. 2015; Chen et al. 2015), ANA (Magorivska et al. 2016) Inflammatory bowel disease: Crohn's disease (Trbojevic Akmacic et al. 2015) Juvenile onset rheumatoid arthritis (Cheng et al. 2017) Antiphospholipid syndrome (Fickentscher et al. 2015) Autoimmune hemolytic anemia—? (Sonneveld et al. 2017b) **Alloimmune diseases** Hemolytic disease of the fetus and newborn—anti-K (Sonneveld et al. 2018) **Infectious diseases** Visceral leishmaniasis (Gardinassi et al. 2014)	**Autoimmune diseases** Autoimmune hemolytic anemia—anti-RBC (Sonneveld et al. 2017b) **Alloimmune diseases** Fetal or neonatal alloimmune thrombocytopenia—anti-HPA (Sonneveld et al. 2016; Wuhrer et al. 2009) **Cancers** Multiple myeloma (Aurer et al. 2007; Fleming et al. 1998) Thyroid cancer (Chen et al. 2012) Lung cancer (Ruhaak et al. 2013) **Infectious diseases** Parvovirus-B19 infection—anti-B19 (Larsen et al. 2020) COVID-19—anti-S (Larsen et al. 2020), anti-N (Larsen et al. 2020) Recurrent respiratory infections (Cheng et al. 2020) RSV infection—anti-RSV (van Erp et al. 2020) HIV infection—anti-HIV (Muenchhoff et al. 2020) Tuberculosis—antigen-specific (Lu et al. 2020)

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Table 13.1 (continued)

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	Tuberculosis (Lu et al. 2016) HIV infection—total (Vadrevu et al. 2018), anti-HIV (Larsen et al. 2020) Meningococcal sepsis (de Haan et al. 2018b) CMV infection — anti-CMV (Larsen et al. 2020) COVID-19 [139] **Cancers** Ovarian cancer (Saldova et al. 2007) Colorectal carcinoma (Theodoratou et al. 2016; Vučković et al. 2016) Malignant hematological diseases[a] (de Haan et al. 2018a) Monoclonal gammopathy of undetermined significance (Bosseboeuf et al. 2017) Multiple myeloma (Bosseboeuf et al. 2017) Bone disease in multiple myeloma (Westhrin et al. 2020) **Other diseases** Alzheimer's disease (Lundström et al. 2014) Chronic kidney disease (Barrios et al. 2016) Type II diabetes (Lemmers et al. 2017) Hypertension (Peng et al. 2019; Gao et al. 2017) Parkinson's disease (Russell et al. 2017) Ischemic stroke (Liu et al. 2018) Hyperuricemia (Hou et al. 2019) Dementia (Zhang et al. 2021)	
F	**Inflammatory diseases and conditions** Inflammation severity (Novokmet et al. 2014) Low back pain (Freidin et al. 2016) **Autoimmune diseases** Systemic lupus erythematosus? (Vučković et al. 2015; Sjöwall et al. 2015) ANCA-associated vasculitis—ANCA (Kemna et al. 2017) Inflammatory bowel disease: ulcerative colitis (Šimurina et al. 2018) Autoimmune thyroid diseases (Martin et al. 2020) Multiple sclerosis (Cvetko et al. 2020) **Alloimmune diseases** Fetal or neonatal alloimmune thrombocytopenia—anti-HPA (Kapur et al. 2014b; Sonneveld et al. 2016; Wuhrer et al. 2009) Hemolytic disease of the fetus and newborn—anti-D (Kapur et al. 2014a), anti-c, anti-E, anti-K (Sonneveld et al. 2017a, 2018) **Infectious diseases**	**Autoimmune diseases** Juvenile onset rheumatoid arthritis (Flögel et al. 1998) Rheumatoid arthritis—total (Gińdzieńska-Sieśkiewicz et al. 2016; Gornik et al. 1999), ACPA (Rombouts et al. 2015) Systemic lupus erythematosus? (Vučković et al. 2015; Sjöwall et al. 2015) ANCA-associated vasculitis (Kemna et al. 2017) Inflammatory bowel disease: Crohn's disease (Šimurina et al. 2018) **Infectious diseases** Visceral leishmaniasis (Gardinassi et al. 2014) Tuberculosis (Lu et al. 2016) HIV infection—total (Vadrevu et al. 2018), anti-HIV (Muenchhoff et al. 2020) **Cancers** Hepatocellular carcinoma (Comunale et al. 2006) Multiple myeloma (Westhrin et al. 2020)

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Table 13.1 (continued)

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	Dengue fever progressing to dengue hemorrhagic fever or dengue shock syndrome—anti-ENV, anti-NS1, anti-HA (Wang et al. 2017) Meningococcal sepsis (de Haan et al. 2018b) HIV infection—anti-HIV (Ackerman et al. 2013; Larsen et al. 2020) CMV infection—anti-CMV (Larsen et al. 2020) Mumps—anti-mumps (Larsen et al. 2020) COVID-19—anti-S (Larsen et al. 2020), anti-N (Larsen et al. 2020) anti-RBD (Chakraborty et al. 2021) Tuberculosis—antigen-specific (Lu et al. 2020) **Cancers** Multiple myeloma (Mittermayr et al. 2017) Malignant hematological diseases[a] (de Haan et al. 2018a) **Other diseases** Dementia (Zhang et al. 2021) Kidney function decline in type II diabetes (Singh et al. 2020) Non-malignant hematological diseases[b] (de Haan et al. 2018a)	**Other diseases** Galactosaemia (Maratha et al. 2016) Hypertension (Gao et al. 2017) Nonalcoholic fatty liver disease (Zhao et al. 2018)
B	**Inflammatory diseases and conditions** IgG4-related disease (Culver et al. 2019) **Autoimmune diseases** Osteoarthritis (Bond et al. 1997) ANCA-associated vasculitis—total (Kemna et al. 2017; Wuhrer et al. 2015), ANCA (Wuhrer et al. 2015) Autoimmune hemolytic anemia—anti-RBC (Sonneveld et al. 2017b) **Alloimmune diseases** Hemolytic disease of the fetus and newborn—anti-c (Sonneveld et al. 2017a) **Infectious diseases** Visceral leishmaniasis (Gardinassi et al. 2014) HIV infection—anti-HIV (Larsen et al. 2020; Muenchhoff et al. 2020) COVID-19—total (Larsen et al. 2020; Petrović et al. 2020), anti-S (Larsen et al. 2020), anti-N (Larsen et al. 2020) **Cancers** Thyroid cancer (Chen et al. 2012) **Other diseases** Hypertension (Wang et al. 2016; Gao et al.	**Inflammatory diseases and conditions** Low back pain (Freidin et al. 2016) Primary sclerosing cholangitis (Culver et al. 2019) COPD (Pavić et al. 2018) **Autoimmune diseases** Rheumatoid arthritis (Pekelharing et al. 1988; Bond et al. 1996, 1997) Juvenile onset rheumatoid arthritis (Bond et al. 1996, 1997) Inflammatory bowel disease: Crohn's disease and ulcerative colitis (Bond et al. 1997) Lambert–Eaton myasthenic syndrome (Selman et al. 2011) Systemic lupus erythematosus (Vučković et al. 2015) Lupus nephritis (Bhargava et al. 2021) **Alloimmune diseases** Hemolytic disease of the fetus and newborn—anti-K (Sonneveld et al. 2018) **Infectious diseases** Infective endocarditis (Bond et al. 1997) Meningococcal sepsis (de Haan et al. 2018b) CMV infection—anti-CMV (Larsen et al.

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Table 13.1 (continued)

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	2017) Galactosaemia (Maratha et al. 2016)	2020) Mumps—anti-mumps (Larsen et al. 2020) Parvovirus-B19 infection—anti-B19 (Larsen et al. 2020) Recurrent respiratory infections (Cheng et al. 2020) Tuberculosis—antigen-specific (Lu et al. 2020) **Cancers** Colorectal carcinoma (Theodoratou et al. 2016) Malignant hematological diseases[a] (de Haan et al. 2018a) **Other diseases** Chronic kidney disease (Barrios et al. 2016) Type II diabetes (Lemmers et al. 2017) Nonalcoholic fatty liver disease (Zhao et al. 2018) Ischemic stroke (Liu et al. 2018) Kidney function decline in type II diabetes (Singh et al. 2020) Dementia (Zhang et al. 2021)
H	**Inflammatory diseases and conditions** IgG4-related disease (Culver et al. 2019) **Infectious diseases** Meningococcal sepsis (de Haan et al. 2018b) **Cancers** Malignant hematological diseases[a] (de Haan et al. 2018a)	
M		**Autoimmune diseases** Multiple sclerosis (Cvetko et al. 2020)

"Down" arrow (↓) refers to a decreased and "up" arrow (↑) to an increased proportion of the corresponding IgG glycosylation trait (as calculated in the corresponding publication) in patients suffering from the disease compared to healthy controls and/or in association with disease activity and severity. In the case of antigen-specific IgG, the arrows refer to the comparison between antigen-specific and total IgG and/or to the association with disease activity and severity. Due to the complexity of IgG glycosylation in a disease setting, the associations shown here are simplified and do not reflect the particulars, such as IgG subclass and clonality, IgG region (total *vs.* Fab *vs.* Fc), analytical methodology, calculation of derived glycosylation traits, subject demographics, clinical parameters, etc. For details, readers are advised to consult the original publications. *G* galactosylated, *S* sialylated, *F* core-fucosylated, *B* bisected, *H* hybrid, *M* high-mannose glycans. *ACPA* anti-citrullinated protein antibody, *ANA* anti-nuclear antibody, *ANCA* anti-neutrophil cytoplasmic antibody, *CMV* cytomegalovirus, *COPD* chronic obstructive pulmonary disease, *COVID-19* corona virus disease 2019, *ENV* envelope protein, *HA* hemagglutinin, *HPA* human platelet antigen, *N* nucleocapsid protein, *NS1* non-structural protein 1, *RBC* red blood cell, *RBD* receptor binding domain, *RSV* respiratory syncytial virus, *S* spike protein. Modified (updated) from our previous review (Gudelj et al. 2018a)—an open-access article, available under the terms of the Creative Commons Attribution License (CC BY): https://creativecommons.org/licenses/by/4.0/

[a]Malignant hematological diseases: acute lymphoblastic leukemia, myelodysplastic syndrome/acute myeloblastic leukemia, acute myeloblastic leukemia

[b]Non-malignant hematological diseases: thalassemia, Fanconi anemia, sickle cell disease, severe aplastic anemia, progressive bone marrow failure, neutropenia congenita, Glanzmann thrombasthenia, hemophagocytic lymphohistiocytosis, X-linked lymphoproliferative disease

in the development of hypertension. Interestingly, the same treatment restored IgG sialylation and reduced tumor load and bone loss in a mouse model of myeloma (Westhrin et al. 2020).

On the level of total serum IgG, increased level of glycosylation of the Fab region observed in some malignant diseases (Zhu et al. 2002, 2003; Radcliffe et al. 2007; Coelho et al. 2010; McCann et al. 2008) is proposed to contribute to disease development and progression by enhancing tumor cell persistence and expansion (Coelho et al. 2010; Amin et al. 2015).

Glycosylation changes on antigen-specific IgG are more likely to be directly involved in disease pathology in case of antibody-mediated auto- or alloimmune diseases or defense from pathogens in case of infectious diseases. The role of differential IgG glycosylation in these cases corresponds to the specifics of a particular disease and the molecular mechanisms underlying its pathology.

In addition to the change in total IgG, multiple infectious diseases are characterized by a distinct glycosylation pattern of relevant antigen-specific IgG in comparison to total IgG (Table 13.1). This implies a distinct regulation of IgG glycosylation, depending on both the disease and the antigen (Ackerman et al. 2013), even within a single individual (Mahan et al. 2016). This supports the notion that IgG glycome relevance should be interpreted in the disease-specific functional context.

One of the rare instances where the role of IgG glycosylation is mechanistically explained is once more linked to the enhanced affinity of afucosylated IgG molecules for FcγRIIIA. In the case of dengue fever, occasionally a secondary, heterologous dengue infection results in severe dengue hemorrhagic fever and dengue shock syndrome. This is attributed to antibody-dependent enhancement (ADE) of the disease by cross-reactivity of afucosylated anti-dengue IgG with platelet antigens, resulting in platelet depletion (Wang et al. 2017). Additionally, the enhanced binding of afucosylated IgG to FcγRIIA and FcγRIIIA promotes the FcγR-mediated viral entry and signaling in cells bearing these receptors on their surface, primarily monocytes and macrophages, resulting in infection progression (Thulin et al. 2020).

A similar relevance for afucosylated antigen-specific IgG is observed in COVID-19 patients. Anti-SARS-CoV-2 IgG with a higher core-fucosylation level is associated with unaided clearance of the infection (Larsen et al. 2020). By contrast, critically ill patients display lower levels of fucosylated anti-SARS-CoV-2 IgG (Larsen et al. 2020; Chakraborty et al. 2021). Furthermore, in in vitro studies afucosylated anti-S/-RBD antibodies were shown to induce enhanced natural killer (NK) cell degranulation (Chakraborty et al. 2021) and elevated production of pro-inflammatory cytokines by primary monocytes and alveolar macrophages, which is likely the background of the severe disease phenotype associated with this glycoprofile in vivo (Larsen et al. 2020; Chakraborty et al. 2021; Hoepel et al. 2020).

Similarly, afucosylated antigen-specific IgG in fetal and neonatal alloimmune thrombocytopenia (FNAIT) and hemolytic disease of the fetus and newborn (HDFN) are thought to contribute, again through enhanced FcγRIIIA-mediated mechanisms, namely phagocytosis and ADCC, to the more severe disease phenotype (Kapur et al. 2014a, b; Sonneveld et al. 2016, 2017a).

In lupus nephritis, a serious complication of SLE, the presence of core fucose was shown to induce upregulated calcium/calmodulin kinase IV expression in podocytes, leading to podocyte injury and limited nephrin synthesis. In the same experimental setting, the presence of terminal galactoses acted protectively (Bhargava et al. 2021).

An interesting recent finding on the importance of Fab glycans emerged in the most explored disease in the context of IgG glycosylation. In RA, a high percentage of anti-citrullinated protein antibody (ACPA) is additionally glycosylated at the Fab region (Rombouts et al. 2016; Hafkenscheid et al. 2017), a feature distinguishing RA patients from $ACPA^+$ but healthy subjects (Kissel et al. 2019; Hafkenscheid et al. 2019). This suggests Fab glycosylation of ACPA might be mechanistically involved in RA development (Rombouts et al. 2016).

13.7 Perspectives for IgG Glycosylation in Precision Medicine

A skewed IgG glycoprofile in comparison to the personal baseline value (requiring longitudinal monitoring) or in comparison to ethnicity-, age-, and sex-matched subjects (requiring a population baseline cohort) in a cross-sectional experimental design, might indicate an increased risk for disease development (Gudelj et al. 2018b), or disease progression (Gudelj et al. 2018a). However, since the alterations in bulk serum IgG glycome composition are not disease-specific, they cannot be used as a stand-alone diagnostic marker. A total IgG glycoprofile of the composition significantly removed from the baseline can instead be used as an indication of a necessity for an examination by an expert clinician.

In case of an established diagnosis, bulk IgG glycome might serve as a predictor of disease progression—e.g., decreased IgG2/3 galactosylation in patients progressing from undifferentiated to rheumatoid arthritis (Sénard et al. 2021). Similarly, IgG glycome is proposed to bear potential for a useful add-on tool for monitoring functional disease progression and response to therapy (Parekh et al. 1988; Kanoh et al. 2004a, 2008; Váradi et al. 2015; Collins et al. 2013; Van Zeben et al. 1994; Rook et al. 1994; Pasek et al. 2006; Gindzienska-Sieskiewicz et al. 2007; Croce et al. 2007; Ercan et al. 2010).

The relevance and biomarker potential of IgG glycome analysis is more evident in some cases of antigen-specific IgG. For instance, due to the increased level of ACPA Fab glycosylation in individuals at risk for RA development, IgG glycome analysis might in the future provide the currently missing understanding (and biomarker) for the first determining pathogenic event leading to disease development (Rombouts et al. 2015; Scherer et al. 2010). Furthermore, as already mentioned, in several diseases a particular antigen-specific IgG glycopattern is associated with a risk for the severe phenotype (Kapur et al. 2014a, b; Sonneveld et al. 2016; Sonneveld et al. 2017a). Similarly, following the mechanistical explanation for the role of afucosylated anti-dengue IgG described in the previous section, afucosylated

maternal anti-dengue IgG is proposed to denote a susceptibility to symptomatic dengue infection in infants (Thulin et al. 2020). The knowledge that a particular glycan profile of antigen-specific IgG, including post-vaccination status for some infectious diseases, is related to the risk of developing (the severe form of) a disease might in the future enable or aid the stratification of patients at risk and timely preventive action.

Another sought-after biomarker type is the one enabling patient stratification aiming at improved differential (sub-)diagnosis and subsequent selection of appropriate therapeutic measures. Differential IgG glycosylation was also suggested as a possibility for such applications. Indeed, the IgG sialylation level predicted response to therapy in Kawasaki disease (Ogata et al. 2013), and the galactosylation level response to anti-tumor necrosis factor (TNF) therapy in RA and Crohn's disease (Váradi et al. 2015), and response to methotrexate therapy in RA (Lundström et al. 2017). Having the means to distinguish non-responders before the very initiation of long and expensive therapeutic treatments is truly an exciting prospect.

In summary, there are multiple possibilities for IgG glycosylation to enter the arena of clinical disease management. Currently, all of the possible applications mentioned here are still at the level of basic research and further studies are necessary to validate the initial findings and propel the IgG glycome analysis to the status of a full-fledged clinical biomarker.

13.8 Conclusions

IgG glycans can modulate virtually all of its numerous effector roles, the specifics depending on the disease and immune context. The associations of multiple IgG glycosylation traits with an immense array of heterogeneous diseases and their different stages imply that there is no single pathway connecting IgG glycome composition and disease development and progression.

Many inflammatory, autoimmune, infectious, cardiometabolic, and neoplastic diseases share a common IgG glycosylation profile of bulk (total) serum IgG, also characteristic for aging and often described as "pro-inflammatory": a decreased level of galactosylated and sialylated glycans, and (sometimes) an increased level of bisected IgG glycans. This pattern is presumably associated with an inflammatory disease component as a part or consequence of disease pathology, or environmental events, such as antigen exposure. It might be mechanistically involved in disease advancement through modulation of inflammation, and, in some cases, manifest before the occurrence of symptoms, thus representing disease predisposition or mark the risk for disease development or progression.

When it comes to a distinct glycosylation profile of antigen-specific versus total serum IgG, IgG glycans are more likely to be directly involved in disease pathogenesis and progression through disease-specific effector mechanisms. This is often the case with afucosylated IgG glycans enhancing the affinity of IgG toward FcγRIIIA.

The read-out of IgG glycosylation has a potential for an (add-on) biomarker helping improve current algorithms for disease prediction and diagnosis, patient stratification, monitoring of disease progression, and response to therapy.

Compliance with Ethical Standards

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Conflict of Interest MP is an employee of Genos Ltd.—a private company that specializes in high-throughput glycomic analysis and has several patents in the field, and of Genos Glycoscience Ltd.—a spin-off of Genos Ltd. that commercializes its scientific discoveries.

Ethical Approval This article does not contain any studies with human participants.

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Chapter 14
Immunoglobulin A Glycosylation and Its Role in Disease

Alyssa L. Hansen, Colin Reily, Jan Novak, and Matthew B. Renfrow

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A. L. Hansen · M. B. Renfrow (✉)
Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, USA
e-mail: renfrow@uab.edu

C. Reily
Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

J. Novak (✉)
Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA
e-mail: jannovak@uab.edu

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Abstract Human IgA is comprised of two subclasses, IgA1 and IgA2. Monomeric IgA (mIgA), polymeric IgA (pIgA), and secretory IgA (SIgA) are the main molecular forms of IgA. The production of IgA rivals all other immunoglobulin isotypes. The large quantities of IgA reflect the fundamental roles it plays in immune defense, protecting vulnerable mucosal surfaces against invading pathogens. SIgA dominates mucosal surfaces, whereas IgA in circulation is predominately monomeric. All forms of IgA are glycosylated, and the glycans significantly influence its various roles, including antigen binding and the antibody effector functions, mediated by the Fab and Fc portions, respectively. In contrast to its protective role, the aberrant glycosylation of IgA1 has been implicated in the pathogenesis of autoimmune diseases, such as IgA nephropathy (IgAN) and IgA vasculitis with nephritis (IgAVN). Furthermore, detailed characterization of IgA glycosylation, including its diverse range of heterogeneity, is of emerging interest. We provide an overview of the glycosylation observed for each subclass and molecular form of IgA as well as the range of heterogeneity for each site of glycosylation. In many ways, the role of IgA glycosylation is in its early stages of being elucidated. This chapter provides an overview of the current knowledge and research directions.

Keywords IgA glycosylation · IgA receptors · IgA1 · IgA2 · *N*-glycosylation · *O*-glycosylation · Mass spectrometry

Abbreviations

C1GalT1	Core 1 β1,3-galactosyltransferase
dIgA	Dimeric IgA
EBV	Epstein–Barr virus
ECD	Electron capture dissociation
ETD	Electron transfer dissociation
FcRL4	Fc receptor-like 4
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GalNAc-T	GalNAc-transferase
Gd-IgA1	Galactose-deficient IgA1

HC	Heavy chain
HR	Hinge region
Ig	Immunoglobulin
IgAN	IgA nephropathy
IgAV	IgA vasculitis
IgAVN	IgA vasculitis with nephritis
LC	Light chain
MBL	Mannose-binding lectin
mIgA	Monomeric IgA
MS	Mass spectrometry
pIgA	Polymeric IgA
RA	Rheumatoid arthritis
SA	Sialic acid
SC	Secretory component
SIgA	Secretory IgA
SLE	Systemic lupus erythematosus
TG2	Transglutaminase 2
V	Variable

14.1 Introduction

Immunoglobulin A (IgA) is one of the five primary immunoglobulins and its production in humans is greater than that of all other immunoglobulins. Humans expend a considerable amount of energy to produce IgA, as ~70 mg of IgA per kilogram of body weight is synthesized daily (Mestecky et al. 1986; Conley and Delacroix 1987). IgA is secreted by IgA-producing cells in two main molecular forms: monomeric IgA (mIgA) and dimeric IgA (dIgA), the latter having a joining chain (J chain) to bind two mIgA molecules. Furthermore, secretory IgA (SIgA) is the main form of IgA found on mucosal surfaces. SIgA has an additional protein chain attached, a secretory component, which is derived from polymeric immunoglobulin (Ig) receptor during transcytosis of dIgA through mucosal epithelial cells. IgA is the second most abundant Ig in the circulation, predominantly as mIgA, (~2 mg/mL), after IgG (~12 mg/mL); however, IgA is the most abundant antibody in external secretions (tears, saliva, colostrum, milk, nasal fluid, gallbladder bile, and intestinal fluid) of mucosal surfaces, secreted locally as SIgA (Jackson et al. 2005). The abundance of IgA highlights the important roles it plays in immune defense processes. Specifically, mucosal surfaces (i.e., respiratory, gastrointestinal, and genitourinary tracts) are often exposed to invading pathogens and IgA acts to protect such vulnerable areas from infection. Equipped with unique structural attributes of its heavy chain and its ability to form monomeric, dimeric, and higher polymeric forms (i.e., three or more mIgA molecules associated with J chain), both circulatory IgA and secretory forms of IgA are capable of neutralizing and removing pathogens

by activating innate and acquired immune functions—blocking pathogens by antigen-specific and nonspecific binding (Russell 2007; Renegar and Small 1994; Phalipon et al. 2002).

Serum IgA is produced predominantly by plasma cells in the bone marrow. It is primarily a monomeric protein with a quaternary structure consisting of two heavy chains (HC) and two light chains (LC) linked by disulfide bonds. In contrast, mucosal IgA, specifically dIgA linked together by the J chain, is produced close to the epithelium by plasma cells in the lamina propria of mucosal surfaces (Koshland 1985). This IgA becomes SIgA when, during transcytosis, the secretory component (SC) is attached. Although most IgA-producing cells are localized in the gastrointestinal system, SIgA is also one of the major antibodies in human tears, saliva, milk, and colostrum (Underdown and Mestecky 1994). Maternal milk is essential for the passive immunity of infants (Newburg et al. 2005; Hanson 1998; Peterson et al. 2013), protecting babies from a variety of infections that otherwise may lead to sepsis and meningitis (Schroten et al. 1998). The diversity of IgA post-translational modifications, due to variable glycosylation, contributes to the effector functions of IgA.

Regardless of the source of IgA, heavy chains of all molecular forms of IgA, as well as the J chain and SC, are glycosylated. In general, glycosylation is the result of a non-template, multi-enzymatic multistep process that ultimately results in a range of glycan heterogeneity. Thus, the post-translationally modified IgA proteoforms are cell type- and lineage-specific. Overall, glycans associated with Igs have been shown to influence antibody functions. Specifically, glycosylation patterns differentially affect the effector functions of Igs (Lin et al. 2015; Li et al. 2017) depending on the type, branching, and modifications of *N*-glycans and/or the terminal sugars of *N*- and/or *O*-glycans, which include galactose and sialic acid (Steffen et al. 2020). In fact, Ig glycosylation can determine whether an antibody glycoform is pro-inflammatory or anti-inflammatory (Li et al. 2017; Pagan et al. 2018). The multiple biological roles of IgA glycans include glycan-mediated antigen-nonspecific binding to bacteria and viruses. The complexity is enhanced by the diverse glycan structures that create a range of heterogeneity.

Herein, we will focus on the glycosylation of human IgA. Specifically, we discuss the location and observed structures at individual amino-acid sites and/or individual clusters of sites. We include a summary of the assessment of native populations of IgA glycans, the range of glycans observed at each site, and how changes in the glycans at a given site have been connected to biological activity and/or diseases.

14.2 IgA Subtypes and Overall Structure

Human IgA exists in two subclasses: IgA1 and IgA2 (occurring in two allotypes—IgA2m(1) and IgA2m(2)) (Fig. 14.1). Each Ig comprises two HCs and two LCs, each with one variable (V) and one (LC) or three (HC) constant domains. Each domain is approximately 110–130 amino acids, averaging 12–13 kDa. Functionally, the

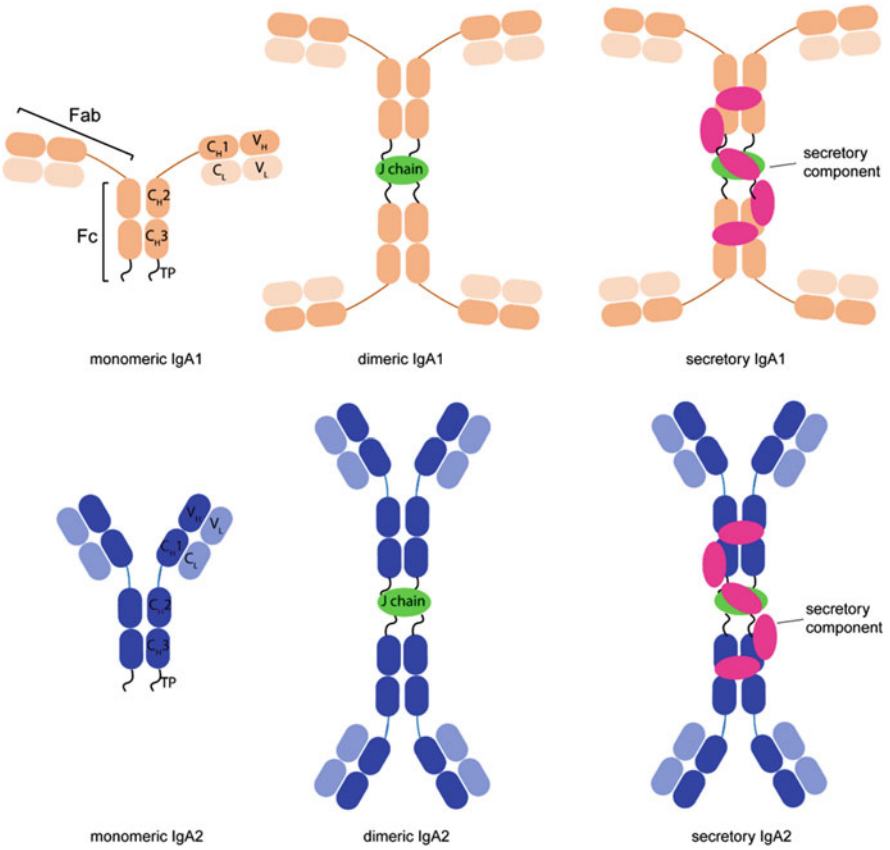

Fig. 14.1 Human IgA. Schematic diagram of IgA1 (top) and IgA2 (bottom) in several molecular forms (monomeric, dimeric, and secretory IgA). Although secretory IgA is depicted as a dimer, larger polymers such as trimers and tetramers can be formed. Heavy chains (V_H, C_H1, C_H2, and C_H3) are shown in orange (IgA1) and blue (IgA2), and light chains (V_L and C_L) are shown in light orange (IgA1) and light blue (IgA2). The tailpiece (TP) is portion of the C_H3 domain that is depicted as an extension. The J chain (green) is present in dimeric and secretory IgA, the secretory component (pink) is a component of secretory IgA

variable regions of HC and LC are responsible for antigen binding, while the constant domains are important for structure/function and defining/modifying the effector functions. Each of the two identical Fab regions of IgA consists of VH and VL and CH1 and CL, with CL being either kappa or lambda. HCs differ within their constant regions encoded by the Cα genes, Cα1 and 2. Each chain begins at its *N*-terminus with the variable region (VH), followed by the constant region, CH1, 2, and 3. CH1 is connected by the hinge region (HR) to the Fc region, consisting of CH2 and 3. Interchain disulfide bridges connect HC and LC of the Fab region and the two HC CH2 domains of the Fc region. Pairings between the collapsed β-barrel domains of adjacent chains (VH with VL, CH1 with CL, and both CH3 domains) are

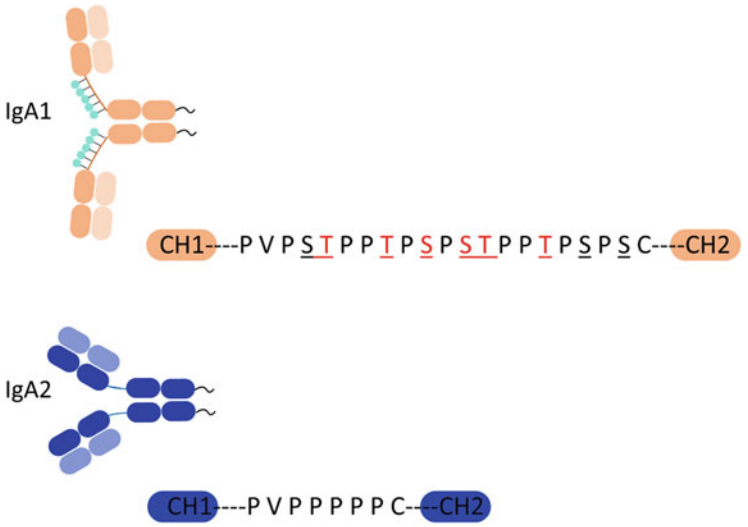

Fig. 14.2 IgA1 and IgA2 hinge regions. Comparison of amino-acid sequences of human IgA1 (top) and IgA2 (bottom) hinge regions. Human IgA1 has nine Ser (S) and Thr (T) amino-acid residues (underlined) in the hinge region segment (between constant regions C_H1 and C_H2 of the heavy chains). Usually, three to six clustered *O*-glycans are attached per hinge region (shown in red). IgA2 hinge region is shorter compared to that of IgA1, does not have Ser and Thr residues and, thus, IgA2 does not have *O*-glycans

mediated through non-covalent interactions, primarily hydrogen bonds and van der Waals contacts. A conserved 18-amino-acid extension of the CH3 heavy chain C-terminus is known as the tailpiece. This feature is unique for IgA and IgM and plays a critical role in dimer and pentamer formation, respectively, though binding with the J chain (Atkin et al. 1996; Yoo et al. 1999).

The HR between the Fab and Fc portions is another feature distinguishing each IgA isotype. IgA1, unlike IgA2, has an HR segment consisting of two octapeptide repeats rich in Ser, Thr, and Pro residues with nine potential *O*-glycosylation sites (Fig. 14.2). Usually, three to six of these sites are *O*-glycosylated (Novak et al. 2012, 2018). In contrast, the IgA2 HR lacks potential *O*-glycosylation sites. Unlike in IgG, IgA HR amino-acid sequence allows for greater flexibility, particularly for IgA1 with its extended sequence. Although this structural characteristic may enhance antigen binding by IgA, it also provides a target for bacterial IgA-specific proteases.

14.3 IgA Glycosylation Sites

The *N*-glycans at individual sites create a range of heterogeneity for both IgA isotypes, as they do for most Igs. Both IgA subclasses carry *N*-linked oligosaccharides. IgA1 and IgA2 have two and four conserved sites of *N*-glycosylation,

respectively, all located in constant regions of the heavy chains. *N*-glycosylation significantly contributes to the total molecular mass of human IgA, accounting for 6–7% of the mass of IgA1, and 8–10% of the mass of IgA2 (Tomana et al. 1976). The IgA subclasses differ from other Igs in both the attachment sites and the overall positions of the *N*-linked glycans and the proximal disulfide bridges. IgA1 and IgA2 possess two similarly located conserved sites of *N*-linked glycosylation. The first site is at Asn144 (IgA1) and Asn131 (IgA2) in the CH2 domain of the heavy chain (the *N*-glycosylation sites herein are indicated by the residue number based on UniProt numbering, IgA1: P01876; IgA2: P01877) (UniProt Consortium 2017). The glycopeptide resulting from trypsin proteolytic digestion, as is conventional for analysis by mass spectrometry (MS), has an identical amino-acid sequence for both isotypes. Thus, if the two isotypes are not separated initially, these two *N*-glycosylation sites are observed as a mixture in standard MS glycosylation analysis (Steffen et al. 2020; Plomp et al. 2018). This is also the case for the second conserved site at Asn340 (IgA1) and Asn327 (IgA2) in the tailpiece. IgA2 has two additional sites of *N*-glycosylation, Asn47 located in the CH1 domain and in the CH2 domain at N205. All adjacent domains (VH with VL, CH1 with CL, and CH3 with CH3) are paired in close proximity to each other, except for the neighboring chains of the CH2 domains, which are not closely aligned. Such non-pairing is a feature also observed in IgG (CH2) and IgE (CH3). The potential solvent exposure, a consequence of the distance between the two CH2 domains, seems to be limited by the presence of *N*-glycans (located at Asn144 in IgA1 and Asn131 and Asn205 in IgA2) which shield the outer surface of the domain. The CH2 domain of IgA1 and IgA2 is stabilized by interchain disulfide bonds formed between three or four cysteine residues (Cys241, Cys242, Cys299, and Cys301) in the upper region of the domain. This feature is in contrast to IgG which instead has several disulfide connections located in the HR (2 HR disulfide bonds for IgG1 and IgG4, 4 for IgG2, and 11 for IgG3) (Liu and May 2012).

The tailpiece extension of the CH3 domain, of both IgA1 and IgA2, has a conserved *N*-glycan located at Asn340 and Asn327, respectively. The tailpiece, similar to the one found in IgM, contains a cysteine residue responsible for polymerization with the J chain. Previous studies have indicated that the tailpiece *N*-glycan of IgA plays an important role in J chain incorporation (Atkin et al. 1996; Sørensen et al. 2000). In plant-produced IgA proteins, the tailpiece is incompletely glycosylated and this deficiency may be the reason for the observed inefficient dimer formation (Göritzer et al. 2017, 2020; Westerhof et al. 2015; Castilho et al. 2018). Recently, it has been demonstrated that the tailpiece of IgA provides an innate line of defense against viruses, with the *N*-glycan mediating such activity (Maurer et al. 2018).

For IgA1, further glycosylation diversity arises through the clustered *O*-glycans attached to the HR. The glycosylated HR is a unique feature to Igs, shared by IgA1 and IgD, and in some forms of IgG3. In IgA1, these clustered *O*-linked glycans are composed of core 1 glycans or terminal or sialylated *N*-acetylgalactosamine (GalNAc), often attached to Thr225, Thr228, Ser230, Ser232, Thr233, and/or Thr236 (amino-acid numbering is based on conventionally used nomenclature for

IgA1 HR). Each Ser/Thr residue can be modified by a GalNAc residue that can be further extended by the addition of galactose (Gal) through a β1,3 glycosidic bond. Up to two sialic acid (SA) residues can be added, one attached to the GalNAc through an α2,6-linkage and the other to Gal by an α2,3-linkage.

14.4 Molecular Forms of IgA and Their Distributions (Monomeric and Polymeric IgA, SIgA, J Chain, Secretory Component)

Unlike most other Ig isotypes, IgA exists in multiple molecular forms (Fig. 14.1). IgA in the circulation is produced mainly in the bone marrow and to a lesser extent in the spleen and lymph nodes. The predominant form of serum IgA is monomeric. IgA destined for mucosal surfaces is produced locally by plasma cells in a polymeric form (pIgA), predominantly dimeric. The ~16-kDa J chain is an additional polypeptide that connects together two or more monomers of IgA via the Fc region tailpiece through disulfide bonds, forming pIgA (Fig. 14.1). Two of the J chain's eight cysteine residues are involved in covalent binding to IgAs tailpiece and the additional six cysteine residues form intramolecular disulfide bridges (Cys12-Cys100, Cys17-Cys91, and Cys108-Cys133) (Bastian et al. 1992). The J chain has a single *N*-linked glycan attached at Asn48. pIgA is formed after glycosylation occurs. Glycosylation is required for dimer assembly, as drastically reduced dimer formation was observed when Asn340 was substituted with alanine to prevent the attachment of *N*-glycans to the tailpiece (Atkin et al. 1996).

SIgA is a multi-polypeptide complex produced in mucosal tissues from pIgA, produced by local plasma cells, undergoing transcytosis through mucosal epithelial cells (Norderhaug et al. 1999). SIgA consists of an SC covalently attached to pIgA (i.e., IgA plus J chain). SIgA is mainly dimeric, although higher oligomers, including trimers and tetramers, are also present. The ~80-kDa SC is comprised of five immunoglobulin-like domains. To form SIgA, a polymeric immunoglobulin receptor-mediated pathway transports pIgA by transcytosis into the mucosal secretions. On reaching the mucosal surface, the polymeric immunoglobulin receptor (pIgR) that binds IgA is cleaved to release SIgA with bound SC which is a proteolytic cleavage product of the pIgR. SC is highly glycosylated by seven *N*-glycans, which contribute up to 25% of its molecular mass (Norderhaug et al. 1999). The SC glycans have several functions, in addition to protecting the SC and the SIgA from proteases (Crottet and Corthésy 1998), they can interact with adhesins and lectins. SC has been shown to bind to a range of bacteria via its glycans (Schroten et al. 1998; Borén et al. 1993; Wold et al. 1990), thereby inhibiting attachment and the subsequent infection of epithelial surfaces. The SC glycans are also involved in the localization of SIgA by anchoring the SIgA to the mucus lining the epithelial surface through its carbohydrate residues.

Circulating IgA is predominantly monomeric and predominantly of IgA1 subclass (~84% IgA1 and ~16% IgA2) (Mestecky et al. 1986), with a small proportion of pIgA (~5–8%) (Delacroix et al. 1982). Dimeric SIgA represents the dominant IgA at almost all mucosal surfaces, although the levels and subclass distributions among the different compartments of the mucosal immune system can vary considerably. For example, in saliva, 90–95% of total IgA is SIgA with a subclass distribution of ~65% IgA1 and ~35% IgA2. This is different for hepatic bile which has a much lower abundance of SIgA (~65%) and a subclass distribution of ~75% IgA1 and ~25% IgA2 (Delacroix et al. 1982). However, even the same compartment, sampled via different methods or times, can provide varying levels of isotype distribution and molecular forms. Furthermore, these distinct differences reflect the dominant Ig source (local production in mucosal tissue versus plasma), the expression of isotype-specific receptors that transport Igs, and the effects of specific regulatory mechanisms (e.g., cytokines and hormones) that influence Ig distribution.

14.5 IgA1 *O*-Glycosylation Pathways

The IgA1 HR with clustered sites of mucin-like *O*-glycosylation represents a unique acceptor site for a post-translational modification process that differs from most other Igs (only IgD has clustered *O*-glycans in its HR). Of the nine potential *O*-glycosylation sites in the HR of human IgA1, usually three to six are *O*-glycosylated (Baenziger and Kornfeld 1974; Mattu et al. 1998; Novak et al. 2000; Renfrow et al. 2007; Takahashi et al. 2010; Hiki et al. 1998; Iwase et al. 1996a; Royle et al. 2003; Franc et al. 2013; Wada et al. 2010a), although glycoforms with up to seven *O*-glycans have been observed in serum IgA1(Pouria et al. 2004). These clustered *O*-glycans of serum IgA1 are usually core 1 glycan (Field et al. 1989; Iwase et al. 1996b). The GalNAc-Gal disaccharide may be sialylated on GalNAc, Gal, or both sugars (Field et al. 1989; Takahashi et al. 2012). *N*-acetylneuraminic acid can be attached to GalNAc through an α2,6-linkage or to Gal by an α2,3-linkage. In addition to the core 1 glycans, some glycans may remain without Gal, i.e., as terminal GalNAc or sialylated GalNAc (Ohyama et al. 2020a, b). Unlike in the process of *N*-glycan biosynthesis, there is no control system for *O*-glycan biosynthesis, and *O*-glycosylation is also not required for folding or export of IgA1 (Gala and Morrison 2002).

Biosynthesis of IgA1 *O*-glycans occurs in the Golgi apparatus of IgA1-producing cells. As for other proteins with clustered *O*-glycans, it is a stepwise process that is not template-driven but rather controlled by expression, activity, and localization of different glycosyltransferases catalyzing the sequential addition of monosaccharides to the acceptor (glyco)protein during its transition through the Golgi apparatus (Reily et al. 2019). The clustered nature of the potential sites of *O*-glycosylation in the IgA1 HR creates a unique amino-acid synthesis template where each step of monosaccharide addition changes the template and has implications for the subsequent steps of monosaccharide addition at adjacent sites. This effect occurs due to

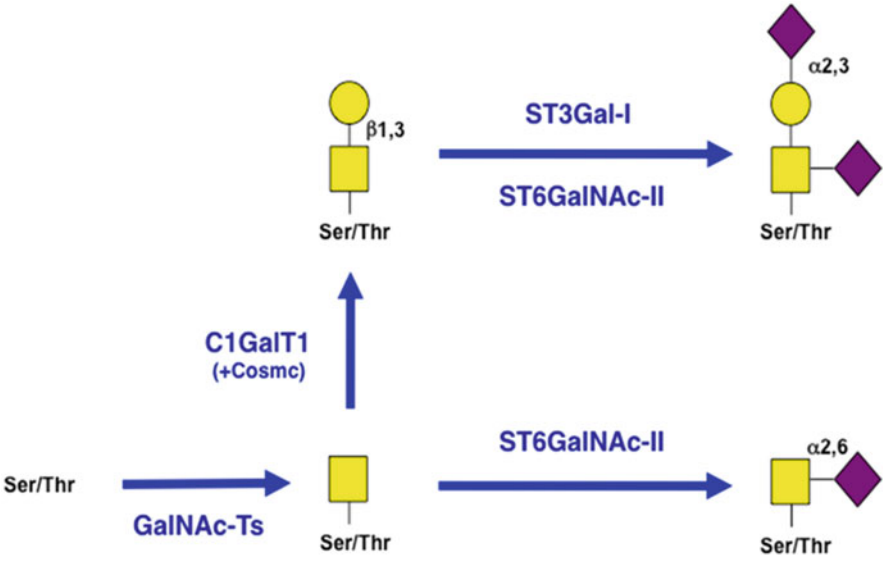

Fig. 14.3 IgA1 *O*-glycosylation pathways. Biosynthesis of IgA1 *O*-glycans occurs in the Golgi apparatus of IgA1-producing cells. The stepwise process begins with the attachment of GalNAc to some of the Ser/Thr residues in the hinge region catalyzed by GalNAc-transferases (GalNAc-Ts). The attached GalNAc residues can be then modified by addition of Gal, mediated by core 1 β1,3-galactosyltransferase (C1GalT1). Production of the active C1GalT1 enzyme depends on its chaperone (C1GalT1C1, Cosmc). The core 1 structures (GalNAc-Gal) of IgA1 can be further modified by sialyltransferases that attach sialic acid to Gal (mediated by an ST3Gal enzyme, e.g., ST3Gal-I) and/or GalNAc residues. Sialylation of GalNAc is mediated by ST6GalNAc-II, as the usual ST6GalNAc-I is not expressed in IgA1-producing cells. Conversely, if terminal GalNAc is sialylated by ST6GalNAc-II, this structure cannot be further modified

some of the glycosyltransferases having glycan-recognizing lectin domains that strongly influence their activity (Stewart et al. 2019, 2021). Also, as more *O*-glycan chains are added and extended in the confined space of the HR, there is a steric hindrance that precludes or inhibits further addition or extension of the clustered *O*-glycans. Still, these physical constraints on the HR create a consistent fidelity of the range and distribution of *O*-glycans that are attached. This is similar to the clustered *O*-glycosylation sites of mucins that have multiple amino-acid tandem repeats as opposed to the two found in IgA1.

IgA1 *O*-glycosylation begins with the attachment of GalNAc to some of the Ser/Thr residues in the HR. In humans, some members of a family of 20 enzymes, GalNAc-transferases (GalNAc-Ts), are involved in this process (Daniel et al. 2020) (Fig. 14.3). It has been proposed that GalNAc-T2 is the main GalNAc-T enzyme responsible for the initiation of *O*-glycosylation of IgA1 (Iwasaki et al. 2003), although other GalNAc-Ts may contribute to the process, including GalNAc-T1, -T11, and -T14 (Stewart et al. 2021; Daniel et al. 2020; Wandall et al. 2007).

GalNAc-Ts determine not only the sites of GalNAc attachment but also the final glycan density, as there are three to six sites per IgA1 HR that are typically

glycosylated. Most GalNAc-T enzymes have two domains, a catalytic domain and lectin domain. Much of our understanding of GalNAc-T enzyme activities comes from studies of purified enzymes, synthetic peptides, glycopeptides, and glycoprotein substrates (Stewart et al. 2019, 2021; de Las et al. 2017). Data from multiple studies point to distinct and complementary roles of catalytic and lectin domains of GalNAc-Ts in the biosynthesis of clustered *O*-glycans of IgA1. Each monosaccharide addition can generate multiple isomers due to the selection of a specific attachment site. Whereas the selection of the first site is driven by the catalytic domain of the GalNAc-T enzyme, the subsequent site selection is substantially impacted by the lectin domain. The glycan density, i.e., the number of GalNAc residues per HR glycopeptide, depends on the expression and activity of GalNAc-Ts isoenzymes that can act on IgA1 and on the selection of the initiation and follow-up sites in the HR. Despite the variability of this step of *O*-glycan biosynthesis, most IgA1 HR glycoforms have three to six *O*-glycans.

The attached GalNAc residues can be modified by addition of Gal, mediated by core 1 β1,3-galactosyltransferase (C1GalT1) (Stewart et al. 2021; Ju et al. 2002, 2006; Aryal et al. 2012). The production of the active enzyme depends on its chaperone (C1GalT1C1, Cosmc) (Ju and Cummings 2002) (Fig. 14.3).

The core 1 structures of IgA1 may be further modified by sialyltransferases that attach sialic acid to Gal (mediated by a ST3Gal enzyme) and/or GalNAc residues (Stewart et al. 2021; Takahashi et al. 2014). Sialylation of GalNAc is mediated by ST6GalNAc-II, as the usual ST6GalNAc-I is not expressed in IgA1-producing cells (Takahashi et al. 2014; Raska et al. 2007; Stuchlova Horynova et al. 2015; Suzuki et al. 2008) (Fig. 14.3). Notably, sialylation of GalNAc on IgA1 by ST6GalNAc-II prevents subsequent galactosylation (Stewart et al. 2021; Stuchlova Horynova et al. 2015; Suzuki et al. 2014). Furthermore, studies with purified enzymes ST6GalNAc-II and ST3Gal-I and partially Gal-deficient IgA1 substrate revealed that prior sialylation by either enzyme influences the activity of the second enzyme (Stewart et al. 2021). These data suggest that the extent of sialylation of the clustered IgA1 HR segment is not only a net result of the enzyme activity but also involves steric hindrances of the clustered *O*-glycans (Novak et al. 2018; Stewart et al. 2021).

Additional knowledge about IgA1 *O*-glycan biosynthesis was obtained from genetic and genomic studies as well as studies of Epstein–Barr virus (EBV)-immortalized IgA1-secreting cells derived from the cells in human peripheral blood (Suzuki et al. 2008). EBV-immortalized IgA1-secreting cell lines derived from healthy individuals and patients with IgA nephropathy (IgAN) provided a new tool for comparative studies of IgA1 *O*-glycosylation in health and disease states. These cell lines produce IgA1 that mimics glycosylation of serum IgA1 of the respective donors (Suzuki et al. 2008). As detailed elsewhere, patients with IgAN have IgA1 in the glomerular immunodeposits and in the circulation enriched for glycoforms with some *O*-glycans deficient in Gal (Novak et al. 2018). Studies of EBV-immortalized IgA1-secreting cell lines from healthy individuals and patients IgAN revealed that dysregulation of expression and activity of several key enzymes is associated with elevated production of Gal-deficient IgA1 (Suzuki et al. 2008, 2014). Specifically, reduced expression/activity of C1GalT1 and its chaperone

Cosmc and elevated expression/activity of ST6GalNAc-II in the cells from IgAN patients are associated with reduced Gal content in the secreted IgA1 (Suzuki et al. 2008).

Studies of familial and sporadic IgAN cohorts revealed heritability of serum levels of Gal-deficient IgA1 (Gharavi et al. 2008; Hastings et al. 2010; Kiryluk et al. 2011). Genome-wide association studies (GWAS) revealed single nucleotide polymorphisms (SNPs) in the noncoding region of C1GALT1, the gene encoding the C1GalT1 galactosyltransferase, associated with serum levels of Gal-deficient IgA1 (Kiryluk et al. 2017; Gale et al. 2017). One of these studies also found associations between SNPs in the noncoding region of C1GALT1C1 (Cosmc), the gene encoding the C1GalT1-specific chaperone, and serum levels of Gal-deficient IgA1 (Kiryluk et al. 2017). siRNA knock-down experiments using immortalized IgA1-secreting cell lines further validated these findings (Kiryluk et al. 2017).

Furthermore, the genetically co-determined Gal content can be further influenced by some cytokines and growth factors, such as interleukin 6 (IL-6), IL-4, and leukemia inhibitory factor (LIF) (Suzuki et al. 2014; Yamada et al. 2010, 2020). This cytokine-mediated overproduction of Gal-deficient IgA1 is due to further dysregulation of expression/activity of specific enzymes (C1GalT1, Cosmc, ST6GalNAc-II) (Suzuki et al. 2014). This process, uniquely enhanced in the IgA1-producing cells from patients with IgAN, is associated with the enhanced and prolonged cytokine signaling, likely due to an aberrant regulation of cellular signaling in JAK-STAT pathways that are engaged by IL-6 or LIF in the cells derived from IgAN patients (Yamada et al. 2017, 2020).

These observed genetic and genomic differences provide points of investigation as to how the final fidelity of IgA1 clustered *O*-glycans can be altered and lead to differences in observed distributions of IgA1 glycosylated proteoforms (IgA1 *O*-glycoforms).

14.6 Assessing Heterogeneity: Analysis of Glycan-Attachment Sites and Range of Heterogeneity

Analysis of IgA1 and IgA2 glycosylation has proven technically challenging due to the multiple molecular forms of IgA, the overall heterogeneity of *N*- and *O*-glycosylation, and the nature of the IgA1 HR where *O*-glycans are attached. Historically, several strategies have been used, including monosaccharide compositional analysis by gas–liquid chromatography (Renfrow et al. 2005) or high-performance anion-exchange chromatography with pulsed-amperometric detection (HPEAC-PAD), Edman sequencing (Baenziger and Kornfeld 1974; Mattu et al. 1998), glycan-specific lectin blotting and ELISA (Moldoveanu et al. 2007), and more recently liquid chromatography-mass spectrometry (LC-MS) (Takahashi et al. 2010). For IgA1 *O*-glycosylation, lectin ELISA has become a standard means to allow high-

throughput analyses in a quantitative manner for serum-based studies (Moldoveanu et al. 2007; Gomes et al. 2010; Moore et al. 2007); however, limitations for several of these methodologies include lack of individual glycoform specificity and the inability to provide information on the sites of attachment and heterogeneity in the context of amino-acid sequence. As a result, much of the standard analysis of IgA glycosylation has moved toward LC-MS.

MS-based techniques utilized to profile glycans and address glycan heterogeneity have become the standard tool for the structural characterization of carbohydrates. Over the last two decades, our understanding of the structures and diversity of glycans in biological samples has increased dramatically. This is the result of the significant improvements in the sensitivity and variety of mass spectrometry approaches. With the help of chromatographic separation, glycan derivatization to improve ionization, and tandem MS fragmentation techniques, oligosaccharide structures and sites of attachment can be elucidated (de Haan et al. 2019). Due to the increased use of IgG as a therapeutic, the majority of analytical development regarding glycosylation has focused on exploring *N*-glycan structures and heterogeneity. While the enzymatic release of *N*-glycans is still a common tool for global *N*-glycan analysis (glycomics), for Ig *N*-glycosylation, amino-acid site-specific analysis has become standard as well as MS analysis of intact proteins to assess the composition and distribution of *N*-glycan heterogeneity (de Haan et al. 2019). Such methodologies that have become standard for the analysis of IgG *N*-glycans are easily applicable to IgA *N*-glycans. As we will discuss more below, that trend has occurred but comparatively there are considerably more studies of *N*-glycans of IgG than IgA. For analysis of IgA *N*-glycans, as discussed above, nearly all sources of IgA in the body have some level of mixture of isotypes and molecular forms. Thus, many of the currently reported analyses of IgA glycosylation are mixtures of IgA produced from multiple sources, however, some groups have begun to make distinctions between various molecular forms.

The features of IgA1 clustered *O*-glycans create a unique analytical challenge. The *O*-glycans are often referred to as "mucin-like" given they mimic *O*-glycans found on the heavily *O*-glycosylated mucin family of proteins that line most epithelial surfaces. Mucins have a large number of tandem repeats that are *O*-glycosylated. The IgA1 HR has only two tandem repeats making it somewhat more amenable to heterogeneity analysis. The *O*-glycans of IgA1 have been implicated in the pathogenesis of IgAN (Tomana et al. 1997, 1999; Novak et al. 2005), and the closely related IgA vasculitis with nephritis (IgAVN) (Allen et al. 1998; Levinsky and Barratt 1979). The goal of identifying differences in IgA1 HR *O*-glycan heterogeneity and composition between patients and normal healthy individuals has driven the development of methodologies. This includes determining the range of IgA1 *O*-glycoforms present and the sites of *O*-glycan attachment defined in the context of adjacent sites as well as the heterogeneity at each individual site (Novak et al. 2018).

As stated, investigators in the IgAN field drove the initial progress in assessing IgA1 *O*-glycosylation. In 1996, Iwase et al. identified two IgA1 HR glycopeptides containing four or five *O*-glycan chains by use of MALDI-TOF mass spectrometry

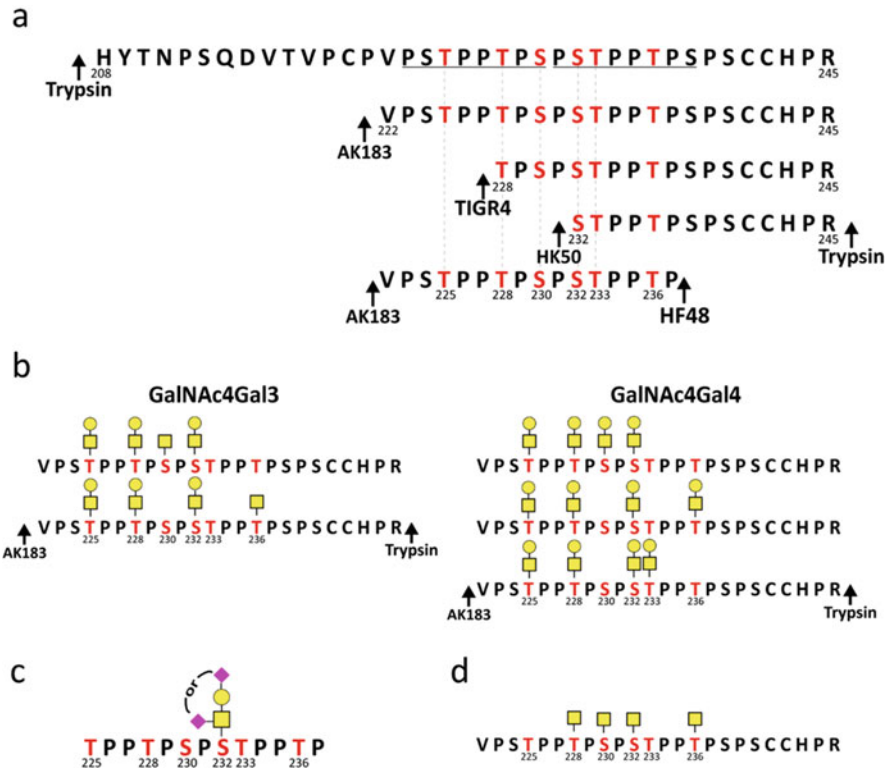

Fig. 14.4 Heterogeneity of IgA1 hinge-region *O*-glycosylation. (**a**) Amino-acid sequence of the hinge region of human IgA1 showing fragments cleaved by IgA-specific proteases (see Fig. 14.6 legend). The six commonly utilized *O*-glycosylation sites are highlighted in red and marked by the numbers below Ser/Thr residues for the AK183-HF48 fragment. The two tandem repeats are underlined in the trypsin fragment (top). (**b**) Examples of two identified IgA1 HR *O*-glycopeptide positional isomers, GalNAc4Gal3 and GalNAc4Gal4 produced by trypsin + AK183 IgA-specific protease. (**c**) Illustration of the potential for sialic acid to be attached to Gal and/or GalNAc residues creating isomers at a single site. (**d**) Four initial sites of GalNAc addition observed by in vitro GalNAc-T2 reactions (Stewart et al. 2019)

(Iwase et al. 1996a). These trypsin-released *O*-glycopeptides were confirmed as the IgA1 HR by sequential glycosidase treatment (neuraminidase, galactosidase, and *N*-acetylgalactosaminidase) and the spectra showed a shift in the peak distribution toward lower masses as carbohydrate residues of the *O*-glycan chains were removed. This promising beginning led to several reports of IgA1 *O*-glycopeptides isolated from serum (Iwase et al. 1998, 1999) and pooled sera of patients with IgAN (Odani et al. 2000), IgA1 isolated from pooled renal biopsies (Hiki et al. 2001), and tonsillar IgA1 (Horie et al. 2003). In 2000, Novak et al. reported the use of IgA-specific proteases that released distinct IgA1 HR fragments (Fig. 14.4) and provided novel means of generating IgA1 HR *O*-glycopeptides for analysis by mass spectrometry and lectins (Novak et al. 2000). This preparation was unique because one of the

IgA-specific proteases (Haemophilus HK50) cleaved between two sites of *O*-glycan attachment (cleavage after Pro231 between Ser230 and Ser232) and therefore provided a general localization of specific *O*-glycan chains N- or C-terminal to the cleavage site.

In terms of IgA1 *O*-glycan site localization, two groups initially identified the predominant sites of *O*-glycan attachment to include Thr225, Thr228, Ser230, Ser232, and Thr236 by use of modified N-terminal sequencing methods (Baenziger and Kornfeld 1974; Mattu et al. 1998). However, these reports analyzed the IgA1 population as a whole with no separation of individual IgA1 *O*-glycopeptides. Later, the introduction of electron radical fragmentation (electron capture dissociation, ECD, and electron transfer dissociation, ETD) in tandem MS lead to the direct assessment of individual IgA1 HR *O*-glycopeptide ions released by trypsin and other proteases, including IgA-specific proteases (Fig. 14.4a). A series of reports by the Renfrow group demonstrated the direct assessment of sites of *O*-glycan attachment for several IgA1 *O*-glycopeptide forms ranging from three to five glycans and having one or two sites comprised of only terminal GalNAc (i.e., galactose-deficient sites-containing IgA1; Gd-IgA1). In the 2012 paper, Takahashi et al. also reported the presence of several IgA1 *O*-glycopeptides that were comprised of isomeric mixtures of equal *O*-glycan chain compositions that were attached at alternate amino-acid sites in the IgA1 HR (Fig. 14.4b) (Takahashi et al. 2012). They also identified a predominant sixth site of *O*-glycan attachment at Thr233. The three attachment sites at Ser230, Thr236, and Thr233 were identified as the predominant sites with galactose-deficient IgA1 (Gd-IgA1) glycans in the IgA1 myeloma protein that was studied as well as two samples of serum IgA1 isolated from healthy individuals. Interestingly, it was the combination of the IgA-specific proteases (Fig. 14.4) plus the novel tandem MS fragmentation that allowed elucidation of many of the details of *O*-glycan attachment that are now considered standard in our understanding of what the heterogeneity of IgA1 *O*-glycans includes. Concurrent with these studies, a study comparing methodologies for assessing *O*-glycans was reported in 2010 using an IgA1 myeloma protein as the reference standard analyzed by fifteen different laboratories (Wada et al. 2010a). While none of the participating laboratories included amino-acid site-specific analysis of IgA1 myeloma reference standard, the study demonstrated that the range of IgA1 *O*-glycopeptides observed was consistent across MS platforms and that there was also a level of consistency in the relative abundance of individual IgA1 *O*-glycopeptides. Since these studies, there has been a steady increase in the use of MS and LC-MS methodologies to assess the distribution of IgA1 hinge-region *O*-glycosylated forms.

One area that remains a challenge is the assessment of IgA1 *O*-glycan sialylation. IgA1 *O*-glycans can be α2,6 sialylated or α2,3 sialylated at any given position. With anywhere from three to six *O*-glycans per hinge region, sialylation significantly increases the number of isomeric mixtures in a population of IgA1 *O*-glycosylated forms (Fig. 14.4c). Takahashi et al. demonstrated the potential for sialic acid isomers at a single site (Thr232). As such, many of the LC-MS analyses have used sialidases to remove the sialic acid residues and reduce the complexity of the IgA1 *O*-glycopeptide mixtures (Takahashi et al. 2010, 2012; Moldoveanu et al. 2007).

The goal of understanding the difference in IgA *O*-glycans in patients with IgAN vs. healthy individuals has led to the development of lectin-based quantitative ELISA tests (Moldoveanu et al. 2007; Zhao et al. 2012). Lectins recognize specific glycan structures and in the case of IgAN, several lectins specific for terminal GalNAc have been utilized in ELISA to provide an IgA1-specific assessment of the extent of Gal-deficient IgA1 in different cohorts. This is done by using IgA from serum or tissue samples and then probing for lectin reactivity (using lectins from *Helix pomatia* agglutinin, HPA or *Helix aspersa* agglutinin, HAA) compared to a reference standard. Through this analysis, investigators have been able to establish the range of Gd-IgA1 levels (based on standardized lectin reactivity) that exists in patients with IgAN, even to the point of utilizing Gd-IgA1 as a marker for IgAN disease progression (Zhao et al. 2012; Maixnerova et al. 2019; Reily et al. 2018). It is possible that LC-MS methodologies may at some point be able to provide a similar output, but currently, these lectin-based ELISA tests have been successfully implemented for larger-cohort studies.

14.7 Observed IgA *N*-Glycosylation Heterogeneity

The characterization and analysis of IgA *N*-glycosylation is a systematic process of localizing the composition and range of glycans linked to asparagine at the glycosylation sites. Assessing Ig *N*-glycosylation has become a common methodology in recent years due to the advent of therapeutic antibodies. This is more the case for IgG, but the principles of systematic mapping and assessment of the range of *N*-glycans at a given site by the use of mass spectrometry have become a tool utilized in the analysis of Igs. For example, these techniques can assess how glycosylation of IgG is affected in different forms, sources, and in responses to specific antigens/vaccines. In the case of IgA, there is the added complexity of assessing the J chain and SC sites of *N*-glycosylation as well as the *O*-glycosylated IgA1 HR (discussed below). To date, comprehensive studies have been performed for mIgA and SIgA from plasma, saliva, and colostrum samples and, in a few cases, these studies have included separation of IgA isotypes. Several different structures of *N*-glycans have been observed, contributing to heterogeneity for both IgA subclasses (Fig. 14.5). Glycan heterogeneity for mIgA and all potential SIgA *N*-glycosylation sites, including the seven on the SC, two on IgA1, four on IgA2, and one on the J chain, are detailed below. Overall, several sources identified IgA *N*-glycans to be mainly of the biantennary complex type, with various levels of galactosylation, sialylation, bisecting GlcNAc, and fucosylation (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Bondt et al. 2016, 2017). The following text provides details of what has been observed to date, but continued experimental analysis of IgA glycan heterogeneity will ultimately aid in understanding the complex biological roles of IgA and SIgA (including SC and J chain) in health and disease.

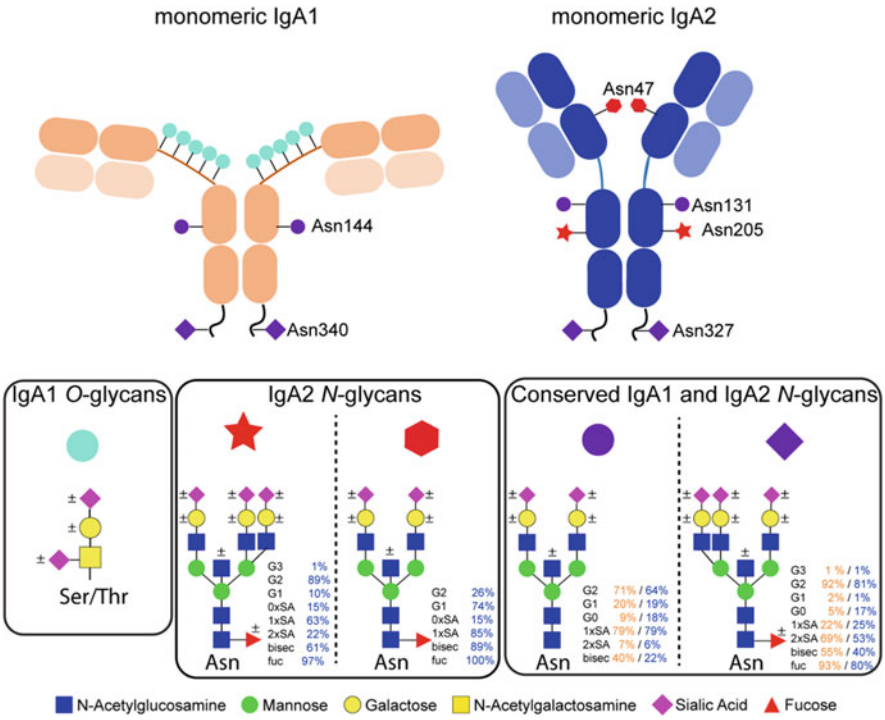

Fig. 14.5 Sites of IgA glycosylation. Illustration of IgA1 and IgA2 and their respective glycosylation sites. IgA1 has three to six observed sites of *O*-glycosylation (shown in cyan) and two IgA1 *N*-glycosylation sites (Asn144 and Asn340, shown in purple). IgA2 has four *N*-glycosylation sites (Asn47 and Asn205, shown in red; and Asn131 and Asn327, shown in purple). The sites in purple are conserved in both IgA subclasses. The outlined area contain representations of the possible *O*- and *N*-glycan structures reported at each site (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Bondt et al. 2016, 2017; Deshpande et al. 2010; Huang et al. 2015; Gomes et al. 2008). The ± symbol indicates the variable presence of each monosaccharide. The relative abundance values (%) are as reported by Steffen et al., who performed site-specific MS quantification of glycans from isolated serum IgA1 and IgA2 (Steffen et al. 2020). Values in orange correspond to IgA1 and values in blue correspond to IgA2

14.7.1 IgA1/IgA2 Asn340/327 N*-Glycosylation*

The *N*-glycosylation sites Asn340 and Asn327 on the tailpiece of IgA1 and IgA2, respectively, are located on tryptic peptides common to both IgA subclasses. Therefore, unless IgA1 and IgA2 are separated (often via jacalin agarose that binds only IgA1), MS analysis will not distinguish between subclasses and information on site-specific glycosylation is gathered for total IgA. At Asn340/327, two different tryptic peptide sequences are commonly identified, the expected LAGKPTHVNVSVVMAEVDGTCY and the truncated LAGKPTHVNVSVVMAEVDGTC. The abundance of each peptide seems to

depend on the origin of IgA, specifically IgA from plasma displays a higher abundance of the truncated peptide while IgA derived from saliva includes higher amounts of the peptide with the C-terminal tyrosine (Plomp et al. 2018). The glycans at this location are primarily biantennary complex-type; however, a low abundance of triantennary glycans (~5%) on the truncated peptide has been reported (Bondt et al. 2016). Additionally, high-mannose and hybrid glycans have been detected in low amounts on the non-truncated peptide (<5% IgA1; <20% IgA2) (Steffen et al. 2020). The relative abundance of bisection, galactosylation, sialylation, and fucosylation are similar for both the non-truncated and truncated peptide. Over 89% galactosylation, sialylation, and fucosylation has been observed for the *N*-glycans of IgA1. Sialylation of IgA1 non-truncated peptide was slightly higher compared to the truncated peptide (89% and 95%, respectively) (Bondt et al. 2016). When observed and reported, the non-truncated peptide on IgA2 displayed slightly lower bisection (<60%) compared to IgA1 (<40%). Fucosylation was also lower in IgA2 (80% IgA2 versus 95% IgA1) (Bondt et al. 2016). In SIgA versus mIgA, Plomp et al. (2018) noted lower fucosylation (1.4-times), galactosylation (2-times), and sialylation (5-times) and higher content of bisecting GlcNac (1.7-times) in salivary compared to plasma IgA. High-mannose glycans were significantly higher in salivary IgA.

14.7.2 IgA1/IgA2 Asn144/131 N-*Glycosylation*

IgA1 Asn144 and IgA2 Asn131 also share the same tryptic amino-acid sequence LSLHRPALEDLLLGSEANLTCTLTGLR, located in the CH2 domain. Analysis has shown that this site is primarily composed of complex-type *N*-glycans, and Plomp et al. (2018) identified approximately 20% high mannose-type glycans and up to 5% hybrid-type/mono-antennary glycans. Steffen et al. (2020) isolated IgA1 and IgA2, specifically identifying non-complex glycan abundance of each subclass at 5% and 20%, respectively. For IgA1, complete galactosylation and approximately 60% sialylation was observed. There was variation in the reported bisecting glycans of Asn144 on IgA1. For example, Bondt et al. reported approximately 25% bisecting glycans which increased to approximately 28% during pregnancy (Bondt et al. 2016). Other studies found it to be higher—between 40 and 50% for IgA1 (Steffen et al. 2020; Plomp et al. 2018). Furthermore, it has been well documented that glycans at Asn144/131 are almost entirely afucosylated (<1%), in contrast to Asn340/327 (Plomp et al. 2018; Bondt et al. 2016). Notable differences in plasma versus salivary IgA were seen for high-mannose glycans, bisecting GlcNAc, galactose, and sialylation (18-times, 1.5-times, 3-times, and 5-times higher in plasma IgA, respectively). This is an interesting finding, as a higher abundance of unprocessed glycans usually reflects rapid processing through the cellular machinery, differential expression of glycosylation enzymes, or restricted access to a specific glycosylation site.

14.7.3 *IgA2* N-*Glycosylation*

For the glycosylation sites Asn47 in the CH1 domain and Asn205 in the CH2 domain, unique to IgA2, *N*-glycans were observed by Plomp et al. on the peptides SESGQNVTAR and TPLTANITK, respectively. The glycans at these sites were mostly complex biantennary (>98% of total abundance) (Plomp et al. 2018). For plasma IgA2, Steffen et al. observed that both glycan sites were mainly monosialylated, had a high level of bisecting GlcNAc, and were almost always fucosylated (Steffen et al. 2020). Differences in galactosylation were observed as Asn47 was mainly monogalactosylated while the antennae of Asn205 were often fully galactosylated. Overall, two- to fivefold differences were observed in the relative abundances of the different glycan types present on plasma vs. salivary IgA2. Specifically, salivary *N*-glycans at both Asn47 and Asn205 showed a higher degree of bisecting GlcNAc and a lower degree of galactosylation, sialylation, and sialylation per galactose compared to plasma *N*-glycans (Steffen et al. 2020; Plomp et al. 2018; Bondt et al. 2016).

14.7.4 *Joining Chain (J Chain)* N-*Glycosylation*

The single *N*-glycosylation site Asn71 on the J chain has been observed as two tryptic peptides: EN_{71}ISDPTSPLR (JC Asn71) and the miscleaved IIVPLNNREN_{71}ISDPTSPLR (JC Asn71m). Plomp et al. (2018) determined that this site contained between 20 and 50% monoantennary and hybrid-type glycans, which is significantly higher than the IgA constant-domain *N*-glycosylation sites. However, differences in glycans in salivary and plasma IgA J chain are consistent with observations for the IgA1 and IgA2 heavy chain *N*-glycosylation sites, namely a higher bisection (3.2 times higher) and lower galactosylation (1.1 times lower) and sialylation (1.7 times lower) in the saliva-derived samples. Surprisingly, the two glycopeptides, JC Asn71 and JC Asn71m, exhibited different glycoprofiles. The miscleaved glycopeptides were more abundant in fucosylation as compared to the expected tryptic glycopeptides (3.2-times higher in plasma and 2.9-times higher in saliva) (Plomp et al. 2018). This observation was previously observed of colostrum, as 10% tryptic and 67% miscleaved glycopeptides were fucosylated (Deshpande et al. 2010). The range of glycan heterogeneity at this site is enhanced as the tryptic glycopeptide exhibits partial or full sialylation, which is uncommon for the miscleaved glycopeptide.

14.7.5 *Secretory Component* N-*Glycosylation*

Seven *N*-glycosylation sites are located on six SC tryptic glycopeptides. *N*-glycans at Asn135, Asn186, Asn421, and Asn469 were determined to be complex-type and biantennary with antennae fully galactosylated and partially sialylated (Plomp et al. 2018; Deshpande et al. 2010). However, other reports showed that sialylation was uncommon for Asn135, Asn186, Asn469, and Asn499 and highest for Asn421 and that Asn135 was abundantly tetraantennary (Huang et al. 2015). Mono-antennary species were identified on Asn499 and the observed glycoforms carried zero to five fucose residues, contributing to both the core and antennary fucosylation. On Asn135, Asn469, and Asn499 between 1 and 4% bisection was observed. All glycosylation sites contained at least five galactose residues and one to three fucose residues.

Glycans Asn83 and Asn90 are located on the same tryptic peptide, complicating site-specific glycosylation analysis. This can be remedied by employing additional proteases. Furthermore, the joint glycan composition $H_{10}N_8F_{2-8}S_{0-3}$ at Asn83 and Asn90, reported by Plomp et al., indicates a similar composition to other SC glycans (Plomp et al. 2018).

14.8 IgA Receptors and Role of Glycans

As detailed above, IgA subclasses are quite diverse in the origin, molecular form, and site-specific glycosylation profiles. The isotype, glycosylation, and molecular form of IgA can impact interactions with various types of IgA receptors. In humans, these receptors include Fc receptors (Fcα-receptor I [FcαRI; CD89] and Fcα/μ receptor [CD351]), polymeric immunoglobulin receptor, transferrin receptor (CD71), lectins (e.g., asialoglycoprotein receptor on hepatocytes), cell-surface galactosyltransferase (e.g., β1,4-galactosyltransferase), and Fc receptor-like 4 (FcRL4, CD307d) (de Sousa-Pereira and Woof 2019; Monteiro and Van De Winkel 2003; Breedveld and van Egmond 2019; Cho et al. 2006; Aleyd et al. 2015; Honda et al. 2016; Yang et al. 2013; Tomana et al. 1993). FcαRI is specific for IgA1 and IgA2 and FcRL4 binds polymeric IgA and IgA found in immune complexes, whereas other receptors can bind other ligands in addition to IgA. Below, we discuss several IgA receptors, focusing on details with respect to glycosylation and molecular forms of IgA.

FcαRI is expressed by myeloid cells, such as monocytes, neutrophils, and some subsets of macrophages and dendritic cells (van Egmond et al. 2001). Serum IgA, predominantly monomeric IgA1, can bind to FcαRI (CD89) (Monteiro et al. 1990; Herr et al. 2003). This receptor plays the main role in the IgA-mediated clearance of pathogens and cancer cells (Woof and Kerr 2006; de Tymowski et al. 2019; Brandsma et al. 2019; Hansen et al. 2018, 2019; Heemskerk and van Egmond 2018). Both the ligand (IgA1 and IgA2) and the receptor are glycosylated (Mattu

et al. 1998; Royle et al. 2003; de Sousa-Pereira and Woof 2019; Aleyd et al. 2015). Whereas IgA *N*-glycans affect IgA thermal stability but not receptor binding (Gomes et al. 2008; Göritzer et al. 2019), *N*-glycans of FcαRI significantly modulate binding affinity to IgA (Göritzer et al. 2019). Furthermore, the binding of IgA1 to FcαRI induces long-range conformational changes in IgA1, propagating up to the *O*-glycosylated HR (Posgai et al. 2018).

FcαRI does not contain any signaling motifs in its cytoplasmic tail and FcαRI-mediated signaling depends on the associated Fc receptor γ chain and its immunoreceptor tyrosine-based activation motifs. FcαRI-mediated activation can induce degranulation, phagocytosis, chemotaxis, and antibody-dependent cellular cytotoxicity. Furthermore, when IgA-FcαRI signaling is combined with activation through pattern recognition receptors (e.g., Toll-like receptors), cytokine production can be induced in antigen-presenting cells. This process is essential for controlling inflammation and inducing both innate and adaptive immune responses. Depending on whether IgA binds to FcαRI in a soluble or aggregated form, it can induce immunosuppressive or pro-inflammatory responses. Soluble forms of IgA in the circulation, monomers and dimers, have low affinity for FcαRI and bind only transiently, thus mediating inhibitory signaling under homeostatic conditions (Hansen et al. 2019). Similar inhibitory effects can be exerted by peptidomimetics and such approaches may be useful to prevent undesirable inflammatory conditions triggered by abnormal IgA-containing immune complexes, such as in IgA-mediated blistering skin diseases (Breedveld and van Egmond 2019; Heineke et al. 2017; Ben Mkaddem et al. 2019).

A recent study underlined differences in the effector functions of human serum IgA1 and IgA2 on myeloid cells. IgA proteins were heat-aggregated or immobilized to mimic immune complexes. Under these conditions, IgA2 was more effective than IgA1 in the induction of pro-inflammatory responses in neutrophils and macrophages (Steffen et al. 2020). However, these differences disappeared after the enzymatic removal of sialic acid or all *N*-glycans from IgA1. Thus, IgA effector functions depend on the subclass and glycosylation, as IgA1 and IgA2 have similar but different glycosylation profiles (IgA2 has 2 additional sites of *N*-glycosylation). Notably, IgA1 contains more sialic acid than IgA2, thus explaining the nature of distinct IgA subclass activities.

Asialoglycoprotein receptor on hepatocytes mediates clearance of IgA (and other asialoglycoproteins) from the circulation (Stockert et al. 1982; Baenziger and Maynard 1980; Baenziger and Fiete 1980; Rifai et al. 2000; Tomana et al. 1988). The binding of IgA is based on recognition of terminal Gal or GalNAc residues by the lectin receptor whereas sialic acid prevents this binding. The IgA molecules internalized by the asialoglycoprotein receptor are degraded and excreted into the bile. This catabolic pathway is thought to explain the relatively short half-life of IgA in the circulation (~5 days).

Polymeric immunoglobulin receptor mediates the specific transport of polymeric immunoglobulins (J-chain-containing polymeric IgA and IgM) across the mucosal epithelium into the secretions. The transport of dimeric IgA across the epithelium (transcytosis) begins with its binding to the polymeric immunoglobulin receptor at

the basolateral surface of the epithelial cell, which is followed by internalization and transport via vesicular compartments to the apical surface of the cell. During the process, the polymeric immunoglobulin receptor is cleaved to release the SC part of the receptor. Secretory component is attached through a disulfide bridge to dimeric IgA, forming secretory IgA released at the apical surface of mucosal epithelium. Notably, SIgA1 exhibits different *O*-glycosylation compared to circulatory IgA1. Circulatory IgA1 contains core 1 *O*-glycans (i.e., GalNAc-Gal that may be sialylated) whereas secretory IgA1 has also core 2 *O*-glycans with extended branches (Royle et al. 2003).

The transferrin receptor (CD71) binds IgA1, but not IgA2, and the IgA1 binding is inhibitable by transferrin (Moura et al. 2001). CD71 binds polymeric, but not monomeric IgA1, and the binding is dependent on glycosylation, namely *O*-glycosylation (Moura et al. 2004). CD71 is thought to participate in the binding of pathogenic IgA1-containing immune complexes by mesangial cells in IgAN (Moura et al. 2004).

FcRL4, expressed by a subset of memory B cells in the epithelia, recognizes polymeric IgA with J chain and heat-aggregated IgA but not secretory IgA (Wilson et al. 2012; Liu et al. 2020). FcRL4 is an inhibitory receptor for IgA. In addition to the four extracellular C2-type Ig domains and a transmembrane domain, it has a cytoplasmic domain that contains three immune-receptor tyrosine-based inhibitory motifs. FcRL4 is thought to regulate B cell responses to mucosal commensal antigens (Liu et al. 2018) and FcRL4-positive B cells have significantly increased usage of the IgA isotype (Amara et al. 2017). FcRL4-positive B cells are also enriched in the joints of patients with rheumatoid arthritis and it is thought that the production of cytokines with bone remodeling activity contributes to the disease pathology (Amara et al. 2017). It is currently not known whether glycosylation of IgA impacts recognition by FcRL4.

These several examples illustrate how glycosylation can impact interactions of different molecular forms of IgA with various types of IgA receptors. Consequently, these glycan-dependent interactions impact the effector function of specific IgA glycoproteoforms.

14.9 Observed IgA1 *O*-Glycosylation Heterogeneity

Based on the involvement of IgA1 in the pathogenesis of IgAN and other similar diseases, considerable progress has been made in the analysis of *O*-glycosylation of serum/plasma IgA1. Much of the current knowledge of IgA1 heterogeneity is based on the analysis of IgA1 myeloma proteins that have been extensively used for the development and comparisons of methodologies discussed above (Renfrow et al. 2005, 2007; Takahashi et al. 2010, 2012). These proteins have also served as model proteins for in vitro and in vivo experiments to understand the pathogenesis of IgAN (Takahashi et al. 2014; Novak et al. 2007, 2011a, b, 2015; Yanagihara et al. 2012; Knoppova et al. 2016; Suzuki et al. 2009, 2019; Rizk et al. 2019; Moldoveanu et al.

2021). Additionally, as the IgA1 HR has only two amino-acid tandem repeats rather than multiple repeats found in mucins, it has been used in many studies as a template for the understanding of clustered *O*-glycan synthesis by glycosyltransferases (Wandall et al. 2007; Ten Hagen et al. 2003; Fritz et al. 2004). This has also contributed to the understanding of what the final IgA1 *O*-glycan heterogeneity is and how it could be altered in a disease such as IgAN.

Nomenclature for IgA1 *O*-glycosylation forms core 1 *O*-glycans is based on glycan composition starting with the first monosaccharide attached, GalNAc, followed by Gal and then sialic acid (SA). A designation of GalNAc4Gal4 implies four *O*-glycan chains per heavy-chain HR comprised of GalNAc + Gal disaccharides. A designation of GalNAc5Gal4 implies five *O*-glycan chains comprised of four disaccharides and a single GalNAc monosaccharide. When sialic acid residues are assigned, such as GalNAc4Gal3SA1 or GalNAc5Gal5SA2, there is no assumption of where or how the SA is linked. IgA1 with at least one GalNAc without Gal in the HR *O*-glycans is often referred to as Gd-IgA1. Among the observed Gd-IgA1 glycoforms, there are variants with one to three Gal-deficient sites per HR. However, it should be noted that these forms are seen in IgA1 from healthy individuals as well as patients with IgAN. Still, most patients with IgAN exhibit elevated circulatory levels of Gd-IgA1.

Early studies of IgA1 from patients with IgAN and healthy controls observed the HR with three to six *O*-glycans (Baenziger and Kornfeld 1974; Mattu et al. 1998; Takahashi et al. 2010, 2012). This distribution has been consistent across many analyses by MS of serum and IgA1 myeloma proteins. There have been some examples of IgA1 myeloma proteins with as few as one to three *O*-glycans per HR, but these appear to be outside the norm (Renfrow et al. 2007). Consistently, the distribution of *O*-glycosylated forms appears to center around those with four (GalNAc4Gal4) and five (GalNAc5Gal4) *O*-glycans (Novak et al. 2000; Takahashi et al. 2010; Iwase et al. 1996a; Wada et al. 2010a; Renfrow et al. 2005). The 2010 comparison study (Wada et al. 2010a) across fifteen different labs confirmed that there was a clear distinction in the abundance of glycoforms no matter what MS technology was used. MS studies of site-specific protein and peptide glycosylation heterogeneity have made use of relative quantitation to reflect the consistent pattern and abundance of glycoform distributions that are observed. Experimentally manipulated changes in abundance are consistent and reflect that the ionization is driven by the peptide portion of the molecule (Stewart et al. 2019). Several groups have demonstrated this for both *N*- and *O*-glycosylation (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Deshpande et al. 2010; Huang et al. 2015) as well as in the analysis of therapeutic glycoproteins (Yang et al. 2016). When IgA1 populations are analyzed with sialidase pretreatment, the distribution is dominated by GalNAc4Gal4 and GalNAc5Gal4 (usually >50% of total combined) followed by a second tier including GalNac4Gal3, GalNAc5Gal3, GalNAc5Gal5, and GalNAc3Gal3 (usually 30–40% of total combined). The remaining distribution is comprised of GalNAc6Gal4, GalNAc6Gal3, GalNac6Gal5, GalNAc3Gal2, GalNAc4Gal2, and GalNAc5Gal2 (each representing <5% of total) (Takahashi et al. 2012; Ohyama et al. 2020b). As discussed more below, most of these identified

IgA1 *O*-glycopeptides are mixtures of amino-acid positional isomers (i.e., glycans attached at variable sites) further complicating the complete assignment of the total distribution of clustered *O*-glycan heterogeneity (Fig. 14.4b).

When viewed in the context of the IgA1 structure, the distribution of *O*-glycosylated forms is a result of the number of available Ser and Thr in the two tandem repeat amino-acid sequence, the enzymatic activity of the glycosyltransferases, and the three-dimensional constraints of the clustered amino-acid sites and flanking cysteines that are involved in disulfide bonds as part of the overall IgA1 quaternary structure. A recent analysis of in vitro reactions of GalNAc-T2 enzyme with IgA1 HR acceptor peptide identified a fast-rate phase of clustered GalNAc addition. For the GalNAc-T2 clustered activity on the native IgA1 HR, the fast phase ends after three GalNAc residues are added, matching the lower end of the predominant IgA1 *O*-glycosylated forms observed in serum IgA1 (Stewart et al. 2019). Interestingly, the natural occurrence of proteoforms with up to six *O*-glycan chains observed for serum IgA1 clustered *O*-glycans is less than what is observed for in vitro synthesis reactions on IgA1 HR peptide substrates where seven and eight additions of GalNAc have been reported (Stewart et al. 2019, 2021). This is where the overall structure of the IgA1 heavy chain and the flanking cysteine residues likely play a role in limiting the final *O*-glycan density. It is also possible that the concurrent addition of Gal and SA to the existing GalNAc residues limits the final heterogeneity as well.

As mentioned above, within this range of three to six *O*-glycans per HR, it is also the presence of amino-acid positional isomers that were initially identified in serum IgA1 (Takahashi et al. 2012). These isomeric mixtures occur from the outset of the synthesis process as demonstrated by in vitro GalNAc-T2 reactions (Stewart et al. 2019). Instead of a strictly ordered process of preferred amino-acid site addition, Stewart et al. demonstrated that there were four alternative initial sites of GalNAc addition at Thr228, Thr236, Ser230, and Ser232 with Thr228 and Thr236 being favored over the other two (Fig. 14.4d) (Stewart et al. 2019). Each initial site of GalNAc addition led to a unique combination of possible second sites of GalNAc addition. While the sites of GalNAc addition were all consistent with those seen with serum IgA1, these experiments revealed multiple GalNAc-addition pathways and explained the biosynthetic origin of isomeric mixtures of IgA1 *O*-glycosylated forms. Additional types of isomers can then occur due to variable sites of the addition of Gal and SA residues. Despite these variable *O*-glycosylation pathways, there is a consistent fidelity of the final IgA1 *O*-glycosylation distribution. For example, Ser230, Thr236, and Thr233 are the sites that show the predominant mixtures of isomers at the disaccharide and monosaccharide levels (Takahashi et al. 2012). These same sites are the predominant sites of Gal deficiency in serum IgA1. A recent study of serum IgA1 used enzymatic removal of GalNAc-Gal disaccharides (Ohyama et al. 2020b). This process resulted in consolidated mixtures of Gd-IgA1 forms with one, two, or three Gal-deficient sites (Ohyama et al. 2020b). These results corroborated Ser230, Thr236, and Thr233 as the predominant sites with Gal-deficient glycans but also indicated low levels of Thr228 and Thr232 with a single GalNAc residue as well. This study thus correlates with the data from in vitro

studies of GalNAc-T2 that revealed multiple isomer possibilities. Since there are other GalNAc-Ts that have demonstrated activity on the IgA1 HR, it is possible that the distribution of IgA1 glyco-isomers is affected by expression patterns of GalNAc-Ts in specific IgA1-producing cells.

O-glycan heterogeneity of IgA1 can be impacted by additional constraints for the addition of Gal and SA. Based on the predominance of GalNAc4Gal4 and GalNAc5Gal4 HR glycoforms, it is logical to conclude the addition of up to four Gal residues is feasible for the C1GalT1 enzyme. The abundance of GalNAc5Gal5 HR glycoforms varies across some reports (~5–10%). This could reflect steric hindrances affecting the addition of a fifth Gal addition and/or a competition with SA addition occurring at the same time. The presence of SA on adjacent *O*-glycan chains has been demonstrated to inhibit C1GalT1 addition of Gal (Stewart et al. 2021; Takahashi et al. 2014; Suzuki et al. 2014). A similar inhibition has been demonstrated for SA addition by ST6GalNAc2 with prior addition of SA by ST3Gal1 and vice versa (Stewart et al. 2021; Suzuki et al. 2014). Interestingly, reports on *O*-glycan heterogeneity of serum IgA1 and IgA1 myeloma proteins have not found more than five SA residues for single IgA1 HR *O*-glycoforms despite there being two potential sites of addition on each glycan of core 1 disaccharide. This could be either the result of the steric hindrance of the clustered *O*-glycans or an artifact of analyzing IgA1 HR by positive-ion mode MS where SA residues add a negative charge. To date, it is unclear if a full accounting of the range of SA in IgA1 *O*-glycans has been accomplished.

In summary, while the generalized IgA1 *O*-glycan heterogeneity is consistent with three to six *O*-glycans per HR, there are underlying isomer mixtures that are likely cell-specific. Additionally, there is ample evidence from lectin ELISA studies that this complex heterogeneity is altered or shifted, reflecting elevated serum levels of Gd-IgA1 in patients with IgAN. The origin of these differences in IgA1 *O*-glycan heterogeneity is an active area of research.

14.10 IgA-Related Diseases

14.10.1 IgA Nephropathy

IgA nephropathy (IgAN) is an autoimmune disease characterized by the glomerular deposition of immune complexes containing galactose-deficient IgA1 (Gd-IgA1) (Knoppova et al. 2016; Rizk et al. 2019). This altered glycosylation results in the increased presence of terminal GalNAc or sialylated-GalNAc in the IgA1 HR. Gd-IgA1 in the glomerular immunodeposits is usually co-deposited with complement C3 and IgG autoantibodies specific to the abnormal glycosylation on the HR of Gd-IgA1. IgAN patients often have elevated levels of Gd-IgA1 and the corresponding IgG autoantibodies in the circulation, leading to the formation of Gd-IgA1-IgG circulating immune complexes. Circulatory levels of Gd-IgA1 are predictive of both disease progression and recurrence after transplantation (Berthoux

et al. 2017). The current hypothesis on the pathobiology of IgAN highlights a four-hit mechanism, where elevated levels of Gd-IgA1 in the circulation (1) coupled with the production of anti-Gd-IgA1 IgG autoantibodies (2) leads to the formation of circulating immune complexes (3) some of which deposit in the glomeruli (4) to induce renal injury (Novak et al. 2008).

The galactose deficiency in IgAN affects HR glycans of the IgA1 and, thus, represents a change in *O*-glycosylation (Fig. 14.3). Gd-IgA1 in the circulation is predominantly in the polymeric (dimeric) form, although monomeric IgA1 is the predominant molecular form in the circulation; mechanisms leading to circulation of this molecular-form-specific effect are not known. In addition to genetically determined serum levels of Gd-IgA1, several studies show that some cytokines can elicit increased Gd-IgA1 production in IgA1-producing cells from IgAN patients. This effect is mediated by reduced expression of the galactosyltransferase encoded by the *C1GALT1* gene, C1GalT1, an enzyme that is responsible for addition of galactose to the GalNAc residues in the HR of IgA1 (Suzuki et al. 2014; Yamada et al. 2017, 2020). Two GWAS studies have found association between IgAN and SNPs in the *C1GALT1* locus, as well as its chaperone protein Cosmc encoded by *C1GALT1C1* (Kiryluk et al. 2017).

The glycosylation of Gd-IgA1 in IgAN exhibits microheterogeneity patterns, both at the level of site attachments and specific glycan composition at each site (Novak et al. 2018; Franc et al. 2013; Ohyama et al. 2020a; Moore et al. 2007). The implication for this variability in glycosylation motifs suggests a semi-stochastic process based on enzyme activities and locations in the Golgi apparatus. Further research is needed to ascertain details of the mechanisms affecting the levels of Gd-IgA1 in the circulation of IgAN patients.

14.10.2 IgA Vasculitis with Nephritis (Formerly Known as Henoch–Schönlein Purpura Nephritis)

IgA vasculitis (IgAV) with nephritis (IgAVN) exhibits kidney-pathology features similar to IgAN, including IgA1 immunodeposits in the mesangium (Selewski et al. 2018). Notable, some patients with IgAVN progress to IgAN. As in patients with IgAN, IgAVN patients have elevated circulating levels of Gd-IgA1 (Kiryluk et al. 2011; Suzuki et al. 2019; Lau et al. 2007; Nakazawa et al. 2019; Pillebout et al. 2017) that form pathogenic immune complexes (Suzuki et al. 2019; Novak et al. 2007). The Gd-IgA1 immune complexes in IgAV typically deposit in the small vessels, leading to systemic vasculitis. In patients with IgAV, only a small fraction develops nephritis, 4–6 weeks from the disease onset, with mesangial proliferation found upon renal biopsy (Lau et al. 2010; Boyd and Barratt 2011). These biopsies show deposition of Gd-IgA1 in a fashion similar to IgAN (Zhao et al. 2020; Sugiyama et al. 2020). This, along with genetic studies showing heritability of circulatory Gd-IgA1 levels in patients with IgAN and IgAVN, indicates a close

relationship between IgAVN and IgAN (Kiryluk et al. 2011; Suzuki et al. 2018; Hastings et al. 2021).

14.10.3 Crohn's Disease

Crohn's disease is a subset of inflammatory bowel diseases, which affects approximately 1% of the US population, and is characterized by inflammation of the gastrointestinal tract (Hanauer 2006). Serological predictors of this disease have been difficult to determine, but recent work on IgA1 HR glycoforms found that decreased number of GalNAc residues per HR was associated with progression vs. recovery in Crohn's patients. Additionally, IgA1 with reduced content of *O*-glycans was found in the inflamed sections of intestinal biopsies from Crohn's patients (Inoue et al. 2012).

14.10.4 Sjögren's Syndrome

Sjögren's syndrome is an autoimmune disease that can affect the tear and salivary glands, with a minority of patients developing other rheumatoid complications, such as systemic lupus erythematosus and rheumatoid arthritis. Patients with Sjögren's syndrome have increased sialic acid content and decreased galactose content on both IgA1 and IgA2 *N*-glycans (Dueymes 1995). Additionally, Sjögren's syndrome patients also have elevated serum levels of IgA1, and follow-up analysis showed that IgA1 was predominantly oversialylated in *N*-glycans (Basset et al. 2000; Levy et al. 1994).

14.10.5 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease where the immune system targets multiple tissues and organs, such as the brain, lung, kidneys, joints, and vasculature. Most SLE patients have a positive anti-nuclear antibody test, but additional tests for anti-ds DNA antibodies, anti-Smith antibodies, and anti-U1RP antibodies can be more specific (Mummert et al. 2018; Olsen et al. 2017). Elevated circulating levels of IgA, 4–6-times higher than in healthy individuals, and abnormal glycosylation of IgA were observed in SLE patients. IgA isolated from female SLE patients showed decreased levels of unbisected biantennary, and tri- and tetraantennary oligosaccharides. Additionally, decreased galactosylation of the HR of IgA1 was found in the same female SLE patients (Matei and Matei 2000).

14.10.6 Rheumatoid Arthritis

The clinical manifestations of rheumatoid arthritis (RA) are swelling and pain in the joints, bone, and cartilage due to inflammation from inappropriate targeting by the immune system. The standard test for RA is a blood analysis of IgG/IgA/IgM antibodies against IgG (RF; rheumatoid factor) and ACPA (anti-citrullinated protein antibodies) (Westra et al. 2021; Kurowska et al. 2017). Mass spectrometry analysis of IgA1 isolated from the circulation of RA patients showed decreased content of GalNAc in the HR of IgA1 (Wada et al. 2010b). Additionally, both IgG and IgA from synovial fluid showed differential galactosylation and sialylation at early and late stages of RA. Terminal α2,6 sialic acid on IgA was lower in early RA compared to advanced RA, while terminal Gal on IgA was lower at early stages of RA and normalized later in the disease (Kratz et al. 2010).

14.10.7 IgA Myeloma

IgA myeloma is a clonal expansion of some IgA-producing cells that undergo genetic mutations leading to uncontrolled proliferation and increased serum levels of IgA (ranging from <30 g/L to >30 g/L) as the disease develops and progresses. Patients with IgA myeloma can develop multiple complications affecting the kidneys and bone and can exhibit various hematological pathologies. Production of IgA from clonal expansion in myeloma was found to include IgA glycoforms that were hyposialylated, which can affect FcαR1 binding (Basset et al. 1999; Bosseboeuf et al. 2020). Studies of the HR *O*-glycans from different IgA1 myeloma proteins have shown ranges of *O*-glycans outside the norm of three to six glycans observed in normal human serum (Renfrow et al. 2007). IgA1 deposition in the kidneys of some patients with IgA myeloma has been found concomitantly with under-galactosylated HRs, similar to the abnormality common to IgAN (Zickerman et al. 2000).

14.10.8 Celiac Disease

Celiac disease is an inflammatory intestinal condition brought on by antibodies targeting the transglutaminase 2 (TG2) protein and gluten-derived TG2-deamidated gliadin peptides. In patients with celiac disease, circulating IgA that targets TG2 has been found to be under-galactosylated, in addition to nonTG2 targeting IgA in circulation (Lindfors et al. 2011; Abbad et al. 2020). CD89 may be involved (Papista et al. 2015) and the interaction of multiple proteins, including CD71, was proposed (Lebreton et al. 2012; Papista et al. 2012).

14.10.9 Complement and IgA

IgA has been considered an anti-inflammatory immunoglobulin (Monteiro 2014; Diana et al. 2013; Ben Mkaddem et al. 2013). Unlike IgG, IgA-mediated activation of complement is not well understood, but it is generally accepted that IgA does not activate the classical complement pathway. However, IgA is thought to be able to activate alternative and lectin pathways of complement, under some circumstances. For example, the less abundant monomeric IgA2 was found to bind mannose-binding lectin (MBL) in ELISA. Additionally, when IgA1 or IgA2, both monomeric and polymeric, were treated with a galactosidase to remove galactose, their binding to MBL increased by over 10-fold (Terai et al. 2006). This would presumably be due to reducing the heterogeneity complexity of *N*-glycans in favor of high mannose glycans such as those observed at Asn340/327 sites. Additionally, IgA1 *N*-glycosylation is critical for binding C3, as evidenced by using point-mutation deletions in critical Asn sites in the Fc region (Chuang and Morrison 1997). This observation was further validated by inhibiting *N*-glycosylation of IgA using tunicamycin and testing for C3 binding (Zhang and Lachmann 1994). Interestingly, sialic acid content was also found to be critical for both IgA1 and IgA2 to fix C3b (Nikolova et al. 1994). In IgAN, where Gd-IgA1 is found in the polymeric form, the polymeric IgA1 is found to be able to bind MBL, resulting in C4 mesangial deposition (Oortwijn et al. 2006; Roos et al. 2001). Of note, complement activation resulting from abnormal IgA1 glycosylation can be found indirectly via anti-Gd-IgA1 IgG autoantibodies in IgAN, where the IgG presumably activates C3 when bound in an immune-complex with Gd-IgA1 (Novak et al. 2015; Knoppova et al. 2016; Rizk et al. 2019; Maillard et al. 2015).

14.11 Infectious Diseases

Some bacteria that can cause human infections utilize tools that negatively affect the defense mechanisms of the host. These tools include IgA-specific proteases and various glycosidases. Whereas the former act exclusively on IgA, the latter affect multiple glycoproteins at the mucosal surfaces. Below, we focus on several aspects of these bacterial virulence factors most relevant to IgA structural integrity and function.

14.11.1 Bacterial IgA-Specific Proteases

Multiple bacterial species that colonize human mucosal surfaces in the oral cavity, digestive tract, respiratory tract, and genital tract produce IgA-specific proteases. These bacteria include streptococci (e.g., *Streptococcus pneumoniae, S. sanguinis,*

Fig. 14.6 Bacterial IgA-specific proteases target the hinge region of IgA1. Amino-acid sequence of the hinge region of human IgA1. Six commonly utilized *O*-glycosylation sites are marked by the numbers below Ser/Thr residues. Numbers 1–5 with arrows above the amino-acid sequence mark peptide bonds that are cleaved by IgA-specific proteases produced by the following species and strains shown as examples: 1, *Clostridium ramosum* AK183; 2, *Streptococcus pneumoniae* TIGR4; 3, *Haemophilus influenzae* HK50; 4, *Neisseria gonorrhoeae* HF13; and 5, *Neisseria gonorrhoeae* HF48

S. mitis, and S. oralis), *Prevotella* and *Capnocytophaga* species and several *Neisseria*, *Clostridium*, and *Haemophilus* species (e.g., *Clostridium ramosum, Neisseria gonorrhoeae, N. meningitidis, Haemophilus influenzae*) (Kilian and Holmgren 1981; Kilian et al. 1979, 1983; Senior et al. 2000; Frandsen et al. 1987, 1995, 1997; Reinholdt et al. 1990; Poulsen et al. 1989, 1996; Lomholt et al. 1992; Lomholt and Kilian 1994; Reinholdt and Kilian 1997; Kosowska et al. 2002; Wang et al. 2020; Chi et al. 2017; Murphy et al. 2015; Janoff et al. 2014; Johnson et al. 2009; Wani et al. 1996).

The bacterial IgA-specific proteases are post-proline endopeptidases that include metalloproteases, serine proteases, and cysteine proteases. These proteases recognize and cleave HR of IgA1, except the protease from *Clostridium ramosum* that can cleave both IgA1 and IgA2, with variable site-specific preferences (Senior et al. 2000; Kosowska et al. 2002; Batten et al. 2003; Mistry and Stockley 2011; Senior and Woof 2005). Figure 14.6 shows examples of commonly recognized sites in IgA1 HR by several representative IgA-specific proteases.

It is thought that bacterial IgA-specific proteases represent a virulence factor for many pathogenic bacteria. Cleavage of IgA1 breaks the Fc fragment from its antigen-binding Fab fragments. This process thus reduces the protective effects of IgA1, such as inhibition of bacterial adherence and antibody complement-dependent killing of bacteria by phagocytic cells. In vitro studies of these proteases have demonstrated that the clustered IgA1 HR *O*-glycans can inhibit cleavage of some proteases indicating a shielding effect of this susceptible region of IgA1 by the *O*-glycans. Various studies examined and confirmed these concepts and searched for IgA-specific protease inhibitors (Wang et al. 2020; Janoff et al. 2014; Weiser et al. 2003; Garner et al. 2013).

14.11.2 Bacterial Glycosidases and Metabolic Utilization of IgA Glycans

Various bacteria occupying mucosal surfaces produce hydrolytic enzymes, such as endo- and exo-glycosidases, able to degrade *O*- and *N*-glycans of mucosal

glycoproteins, including IgA. For example, in bacterial vaginosis, a common polymicrobial imbalance of the vaginal flora, IgA in the secretions is subjected to stepwise deglycosylation and subsequently enhanced proteolysis. These modifications are thought to compromise the ability of IgA to neutralize and eliminate pathogens (Lewis et al. 2012; Robinson et al. 2019; Govinden et al. 2018; Moncla et al. 2016; Smith and Ravel 2017). *Streptococcus pneumoniae* is a major airways pathogen that can produce multiple exo- and endoglycosidases to degrade *O*- and *N*-glycans of IgA and other glycoproteins (King et al. 2006; Marion et al. 2009; Syed et al. 2019; Robb et al. 2017; Blanchette et al. 2016; Kahya et al. 2017). For *S. pneumoniae* colonization of the upper airways, neuraminidase NanA is essential (Brittan et al. 2012). In some instances, a synergy between *S. pneumoniae* and influenza A sialidases can impact nasal colonization and middle ear infection by pneumococci (Wren et al. 2017). Conversely, IgA glycans, specifically sialic acid of the tail *N*-glycans, can inhibit influenza A and other enveloped viruses that use sialic acid as a receptor (Maurer et al. 2018). Another example of pathogenic bacteria utilizing glycosidases includes periodontitis and the associated bacteria, such as *Porphyromonas gingivalis* known to target host sialylated glycoproteins for immune dysregulation (Sudhakara et al. 2019). In the gastrointestinal system, IgA has multiple functions and glycans of secretory IgA1 (SIgA) are known to mediate the binding of SIgA to the microbiota. In fact, it has been proposed that IgA1 *N*- and *O*-glycans provide a link between innate and adaptive immune systems that sustain intestinal homeostasis (Royle et al. 2003; Reily et al. 2019; Mathias and Corthésy 2011a; Pabst and Slack 2020; Gupta et al. 2019; Nakajima et al. 2018; Corthésy 2013; Mathias and Corthésy 2011b). The net result of releasing the glycans is not only immune dysregulation and immune evasion but also a metabolic advantage. Some bacteria can metabolize the released glycans to support their growth (Perman and Modler 1982; Garbe and Collin 2012; Sjögren and Collin 2014).

14.11.3 IgA as a Potential Therapeutic Antibody

As outlined earlier, functions of human IgA subclasses, IgA1 and IgA2, are impacted by multiple aspects, including glycosylation, that directly and indirectly regulate IgA effector functions. For example, IgA1, but not IgA2, has *O*-glycans in addition to *N*-glycans and is more sialylated at the *N*-glycans. Consequently, IgA2 can induce pro-inflammatory responses in neutrophils and macrophages more effectively than IgA1 (Steffen et al. 2020). Conversely, polymeric IgA1, but not polymeric IgA2 or monomeric versions of either subclass, interacts with transferrin receptor CD71, apparently through IgA1 HR *O*-glycans (Moura et al. 2004). Furthermore, IgA glycans, specifically Gal and GalNAc, impact catabolism of IgA in the liver, a process that depends on the asialoglycoprotein receptor on hepatocytes and contributes to the short half-life of IgA in the circulation.

These aspects are at the forefront of recent research due to a growing interest in using IgA as a therapeutic antibody. The therapeutic applications of IgA may include

systemic and mucosal delivery. In either setting, a better understanding is needed about the impact of producing-cell-specific glycosylation on IgA effector functions, receptor interactions, and pharmacokinetics. Biotechnological systems for the production of recombinant IgA, as well as other recombinant Igs, can include plants (Göritzer et al. 2017, 2019, 2020; Strasser et al. 2021; Kallolimath et al. 2016, 2020; Montero-Morales et al. 2019; Montero-Morales and Steinkellner 2018; Kallolimath and Steinkellner 2015). A better understanding of the differential glycosylation of various molecular forms and subclasses of IgA is needed to inform design and production of recombinant therapeutic IgA for future preclinical and clinical trials.

14.12 Therapeutic IgA Antibodies

Monoclonal antibody therapeutics have gained popularity in the medical field to treat a large set of pathologies due to their intrinsic specificities to target antigens. These therapies have largely been using IgG, although some research has been performed in vivo and in vitro to assess the feasibility of IgA therapeutics. IgA can be both pro-inflammatory and anti-inflammatory depending on the structure of its glycans which can regulate its binding to the FcαR1 and FcαR2 receptors. For example, treatment with IgA monoclonal antibodies against human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) enhanced antitumor activity in FcαR1 transgenic mice (Boross et al. 2013; Meyer et al. 2016). This is thought to be done via activation of neutrophils and macrophages, which can be activated through IgA binding to FcαR1 and activating the immunoreceptor tyrosine-based activation motifs (ITAMs) pathway (Heineke and van Egmond 2017).

Targeting FcαR1 could prove to be an important clinical modality for IgA monoclonal treatments. However, a number of hurdles need to be overcome for IgA therapies to work. IgA is cleared more rapidly than IgG from the circulation in part due to asialoglycoprotein receptor expression in the liver. This receptor can bind multiple glycosylation sites on the IgA molecule, which can be inhibited by glycoengineering IgA to have increased terminal sialic acids (Rifai et al. 2000; Boross et al. 2013; Meyer et al. 2016). Interestingly, decreasing sialic acid content of IgA increases its pro-inflammatory capabilities (Steffen et al. 2020), leading to a trade-off between circulatory residence time (bioavailability) and the desired effector function of monoclonal IgA generated.

Production of properly folded and glycosylated IgA proteins can be difficult in mammalian systems, especially tuning the glycoengineering component to create the needed structures. This is in part due to the many different forms of *N*- and *O*-glycans that can be produced by mammalian cells, which makes it difficult to generate consistent glycoforms on a complex background (Montero-Morales and Steinkellner 2018). A more understudied way of generating consistent glycosylated proteins is to use modified plant cells that have undergone genetic modifications to specific glycosyltransferases involved in *N*- and *O*-glycosylation pathways. This approach was recently used successfully to generate IgG monoclonal therapies

against the Ebola virus outbreak in Africa (Qiu et al. 2014; Davey et al. 2016). The generation of mammalian-like *N*-glycosylation-competent plants for IgG antibodies was first done in the *Nicotiana benthamiana* species, using genetic manipulations to inactivate the endogenous β1,2-xylosyltransferase (XylT) and α1,3-fucosyltransferase (FucT) genes (ΔXT/FT). This produced a biologically competent anti-HIV monoclonal antibody, with the same glycosylation as those expressed in Chinese hamster ovary (CHO) cell lines (Strasser et al. 2008). These ΔXT/FT plants have now been deployed to produce normally *N*- and *O*-glycosylated IgA1. While the whole IgA1 protein was not reproduced in the ΔXT/FT variant, the researchers were able to overcome hydroxyproline formation at the HR by overexpressing GalNAc-T2. This produced a variant of the core-1 motif-containing galactose, and only modest sialylation (Dicker et al. 2016). These studies that generate the technology for robust manufacturing of glycosylation competent IgA will be able to open up a larger sector for IgA therapeutics.

14.13 Conclusions

Understanding IgA glycosylation and its role in disease is still a developing field of study. However, with the help of MS, and recent technological advances, substantial progress has been made. Although still in its early stages, it is becoming more feasible to evaluate and decipher glycan structures and range of heterogeneities, especially when the different IgA isotypes and molecular forms are separated. Our knowledge of the role of IgA glycosylation in various diseases is currently based on observational studies. The one exception is the role that IgA1 *O*-glycans have been shown to play in the pathogenesis of IgA nephropathy. As the role of IgA in the immune response is further delineated from other immunoglobulins, the role of the glycans, especially those unique to the various IgA isotypes and molecular forms will be better understood. This will likely lead to IgA being more frequently used as a therapeutic to target specific disease states and/or locations within the human body.

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Compliance with Ethical Standards

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Conflict of Interest MBR and JN are co-founders and co-owners of and consultants for Reliant Glycosciences, LLC. MBR and JN are co-inventors on US patent application 14/318,082 (assigned to UAB Research Foundation). ALH and CR declare no conflict of interest.

Ethical Approval Not applicable, as this is a review article and, thus, there are no human subjects recruited for this study or any animals used.

Informed Consent Not applicable, as this is a review article and, thus, there are no human subjects recruited for this study.

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Part IV
Applications

Chapter 15
Importance and Monitoring of Therapeutic Immunoglobulin G Glycosylation

Yusuke Mimura, Radka Saldova, Yuka Mimura-Kimura, Pauline M. Rudd, and Roy Jefferis

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Y. Mimura (✉) · Y. Mimura-Kimura
Department of Clinical Research, National Hospital Organization Yamaguchi Ube Medical Center, Ube, Japan
e-mail: mimura.yusuke.qy@mail.hosp.go.jp

R. Saldova
NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Blackrock, Dublin, Ireland

UCD School of Medicine, College of Health and Agricultural Science, University College Dublin, Belfield, Dublin, Ireland

P. M. Rudd
NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Mount Merrion, Blackrock, Dublin, Ireland

Bioprocessing Technology Institute, Agency for Science, Technology and Research, Centros, Singapore

R. Jefferis
Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

M. Pezer (ed.), *Antibody Glycosylation*, Experientia Supplementum 112,
https://doi.org/10.1007/978-3-030-76912-3_15

Abstract The complex diantennary-type oligosaccharides at Asn297 residues of the IgG heavy chains have a profound impact on the safety and efficacy of therapeutic IgG monoclonal antibodies (mAbs). Fc glycosylation of a mAb is an established critical quality attribute (CQA), and its oligosaccharide profile is required to be thoroughly characterized by state-of-the-art analytical methods. The Fc oligosaccharides are highly heterogeneous, and the differentially glycosylated species (glycoforms) of IgG express unique biological activities. Glycoengineering is a promising approach for the production of selected mAb glycoforms with improved effector functions, and non- and low-fucosylated mAbs exhibiting enhanced antibody-dependent cellular cytotoxicity activity have been approved or are under clinical evaluation for treatment of cancers, autoimmune/chronic inflammatory diseases, and infection. Recently, the chemoenzymatic glycoengineering method that allows for the transfer of structurally defined oligosaccharides to Asn-linked GlcNAc residues with glycosynthase has been developed for remodeling of IgG-Fc oligosaccharides with high efficiency and flexibility. Additionally, various glycoengineering methods have been developed that utilize the Fc oligosaccharides of IgG as reaction handles to conjugate cytotoxic agents by "click chemistry", providing new routes to the design of antibody-drug conjugates (ADCs) with tightly controlled drug-antibody ratios (DARs) and homogeneity. This review focuses on current understanding of the biological relevance of individual IgG glycoforms and advances in the development of next-generation antibody therapeutics with improved efficacy and safety through glycoengineering.

Keywords Critical quality attribute · Drug-antibody conjugate · Endoglycosidase · Glycoengineering · Glycoform · Glycoprotein · Oligosaccharide · Recombinant antibody therapeutics

Abbreviations

2-AB	2-aminobenzamide
ACPA	Anti-citrullinated protein antibody
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary
CQA	Critical quality attribute
DAR	Drug-antibody ratio
DBCO	Dibenzoazacyclooctyne
ENGase	Endoglycosidase
FcγR	Receptor for Fc portion of IgG
FcRn	Neonatal Fc receptor
HILIC	Hydrophilic interaction liquid chromatography

IVIG	Intravenous immunoglobulin
PR3-ANCA	Proteinase 3-anti-neutrophil cytoplasmic antibody
RhIG	Anti-RhD IgG
RSV	Respiratory syncytial virus
sFcγRIIIa	Soluble form of FcγRIIIa
SG-Ox	Sialoglycan-oxazoline
SGP	Sialylglycopeptide

15.1 Introduction

Glycosylation of a glycoprotein is a complex and extensive posttranslational modification that influences protein conformation, stability, solubility, pharmacokinetics, biological activities, and immunogenicity (Mimura and Jefferis 2021; Rudd and Dwek 1997; Varki et al. 2017). The oligosaccharides attached at Asn297 residues of IgG-Fc are heterogeneous due to variable addition and processing of outer-arm sugar residues (sialic acid, galactose, and bisecting GlcNAc) and fucose onto the core diantennary heptasaccharide ($GlcNAc_2Man_3GlcNAc_2$, designated G0) (Arnold et al. 2006; Jefferis 2016, 2017c; Mimura and Jefferis 2021) (Fig. 15.1) and subject to alteration dependent on the expression hosts and culture conditions. The differentially glycosylated species (glycoforms) of IgG-Fc express unique biological activities, modulating antibody effector functions including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and may be potentially immunogenic when bearing nonhuman oligosaccharides including α(1–3)-galactose and/or N-glycolylneuraminic acid (Cobb 2020; Mimura et al. 2018; de Taeye et al. 2019). Due to the profound impact of mAb glycoform on biological activity and clinical outcome, the glycoform profile is a CQA for each mAb and is characterized by state-of-the-art analytical methods to demonstrate consistency and comparability between batches, and biosimilarity between biosimilar and innovator products (Jefferis 2017a, b; Beck and Liu 2019; De Leoz et al. 2020) (Fig. 15.1).

Whether elevated serum levels of agalactosylation (G0) of IgG-Fc in certain chronic inflammatory diseases are associated with the disease activity remains a long-standing question. Site-specific glycosylation analyses of IgG-derived glycopeptides have revealed differences in galactosylation, sialylation, fucosylation, and bisection of glycoform profiles between autoantibody (antigen-specific IgG) and total (bulk, nonspecific) IgG (Kemna et al. 2017; Wuhrer et al. 2015). A new role of Fc glycosylation of total IgG has been proposed in which increased galactosylation/sialylation of total IgG results in diminished FcγR activation status in immune effector cells through increased FcγR occupancy (Dekkers et al. 2018), which provides insights into the generation of anti-inflammatory antibody therapeutics via glycoengineering.

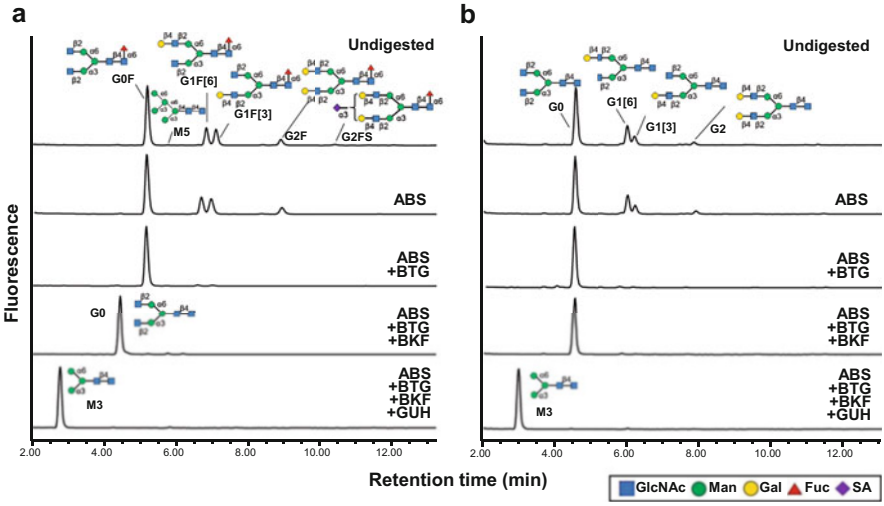

Fig. 15.1 Oligosaccharide profiles of mAbs by hydrophilic interaction liquid chromatography (HILIC). (**a**) Nivolumab (human anti-PD-1 IgG4), (**b**) Mogamulizumab (humanized anti-CCR4 IgG1). The oligosaccharides were released with peptide-*N*-glycosidase F from the heavy chains of the mAbs in the SDS-PAGE gel bands and labeled with 2-aminobenzamide (2-AB) as previously described (Royle et al. 2006). The fluorescently labeled oligosaccharides were separated by ultraperformance liquid chromatography (UPLC) on a sub-2μm hydrophilic interaction-based stationary phase with a Waters Ethylene Bridged Hybrid (BEH) Glycan chromatography column (150 × 2.1 mm i.d., 1.7μm BEH particles) (Bones et al. 2010; Doherty et al. 2012). The oligosaccharide peaks were assigned in accordance with the previous study (Pucic et al. 2011). 2-AB-labeled oligosaccharides were digested using arrays of the following enzymes: *Arthrobacter ureafaciens* sialidase (ABS), bovine testis β-galactosidase (BTG), bovine kidney α-fucosidase (BKF), β-*N*-acetylglucosaminidase cloned from *Streptococcus pneumoniae*, expressed in *Escherichia coli* (GUH)

Various glycoengineering approaches have been developed to produce nonfucosylated mAbs because of the importance of ADCC for the treatment of cancers, inflammatory diseases, and infection. As of 2020, four nonfucosylated mAbs have been approved for treatment of cancers, asthma, or autoimmune diseases, and currently >30 glycoengineered mAbs are under clinical evaluation (Lu et al. 2020; Kaplon et al. 2020; Pereira et al. 2018). Recently, the endoglycosidase-based chemoenzymatic glycoengineering approach has been introduced for remodeling of IgG-Fc glycosylation with preassembled oligosaccharides. This method allows for the preparation of potentially any homogeneous IgG-Fc glycoform, including those bearing fully sialylated, bisected, multi-antennary complex-type, oligomannose-type, or hybrid-type oligosaccharides (Wang et al. 2019). Furthermore, in vitro glycoengineering approaches are also exploited for the preparation of ADCs with tightly controlled drug-antibody ratios (DARs) and homogeneity. Production of selective IgG glycoforms via glycoengineering is a promising means to tailor the clinical efficacy of mAbs. This review provides an overview of

the current understanding of glycobiology of natural IgG and mAbs that may be exploited to develop next-generation antibody therapeutics with optimized activities via glycoengineering.

15.2 Influence of Fc Glycosylation on the Structure and Function of IgG-Fc

Glycosylation of IgG-Fc has been shown to be essential for optimal expression of biological activities mediated through FcγRs (FcγRI, FcγRIIa/b/c, FcγRIIIa/b) and the C1q component of complement, and deglycosylated IgG is deficient in binding or activating these effector ligands. The IgG-Fc crystal structure reveals that the oligosaccharides are integral to the protein structure and sequestered within the internal space enclosed by the two C_H2 domains (Deisenhofer 1981). The electron density map provides coherent diffraction for the mono-galactosylated oligosaccharide and allows the possibility of >70 non-covalent interactions with 14 amino acid residues of the C_H2 domain (Fig. 15.2) (Deisenhofer 1981; Padlan 1990).

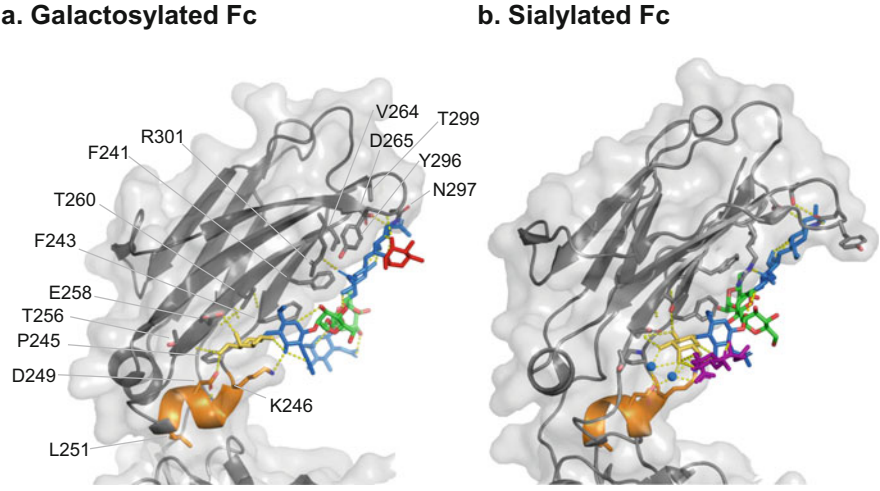

Fig. 15.2 Contacts between the Asn297-linked oligosaccharide and amino acid residues in the C_H2 domain of human IgG-Fc. Crystal structures of the C_H2 domains of monogalactosylated IgG-Fc (PDB ID code: 1Fc1) (**a**) and disialylated IgG-Fc (PDB ID code: 5GSQ) (**b**). GlcNAc, fucose, mannose, galactose, and sialic acid are shown in blue, red, green, yellow, and purple, respectively. Hydrogen bonds are shown with yellow dashed lines. Note that the terminal galactose is hydrogen-bonded with Asp249 (**a**) while the terminal sialic acid and galactose interact with Asp249 via water-mediated hydrogen bonds (water: blue sphere, **b**). The α-helix from Lys246 to Leu251 at the C_H2/C_H3 interface is shown in orange. The molecular models were produced with PyMOL (The PyMOL Molecular Graphics System, Version 2.1.0, Schrödinger, LLC)

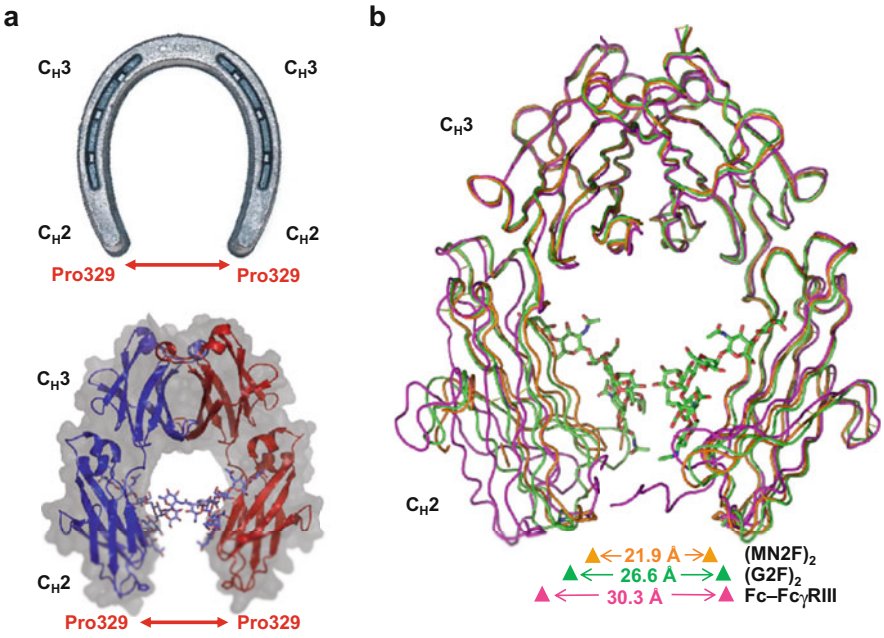

Fig. 15.3 The IgG-Fc horseshoe model (**a**) and superimposed image of IgG-Fc glycoforms $(G2F)_2$ and $(MN2F)_2$ and IgG-Fc complexed with a recombinant soluble form of FcγRIII (sFcγRIII) (**b**). The G2F oligosaccharides are shown in green sticks. The Pro329 residues located in the FG loop of the C_H2 domains of $(G2F)_2$, $(MN2F)_2$ glycoforms, and IgG-Fc complexed with sFcγRIII are indicated by green, orange, and pink arrowheads, respectively (PDB ID codes: 1H3V, 1H3T, and 1E4K) (Krapp et al. 2003; Sondermann et al. 2000)

Following successive truncation of non-reducing Fc sugar residues, the thermal stability of the C_H2 domains was progressively decreased while the C_H3 domains were unchanged as revealed by differential scanning microcalorimetry. The fully galactosylated $(G2F)_2$ Fc glycoform exhibited a higher enthalpy change (ΔH) for the unfolding of the C_H2 domains than the $(G0F)_2$ glycoform, indicating that terminal galactose residues can confer stability on the C_H2 domains (Ghirlando et al. 1999). Removal of terminal GlcNAc from the $(G0F)_2$ glycoform resulted in a marked reduction of thermal stability, due to loss of the interactions with the side chain residues of Phe243, Lys246, and Thr260. Subsequent removal of the branch mannose residues, yielding the trisaccharide GlcNAc-GlcNAc-Man, did not result in further loss of stability but binding to a soluble form of FcγRIIb was markedly reduced (Mimura et al. 2000, 2001).

Tracing the α-carbon chain amino acid sequence of the IgG-Fc, as determined by X-ray crystallography, yields a "horseshoe" structure (Fig. 15.3a), in which the C_H3 domains form the "head" and the C_H2 domains are distanced from each other to accommodate the oligosaccharide. Removal of the oligosaccharide results in partial closure of this "open" structure with consequent loss of FcγR and C1 binding

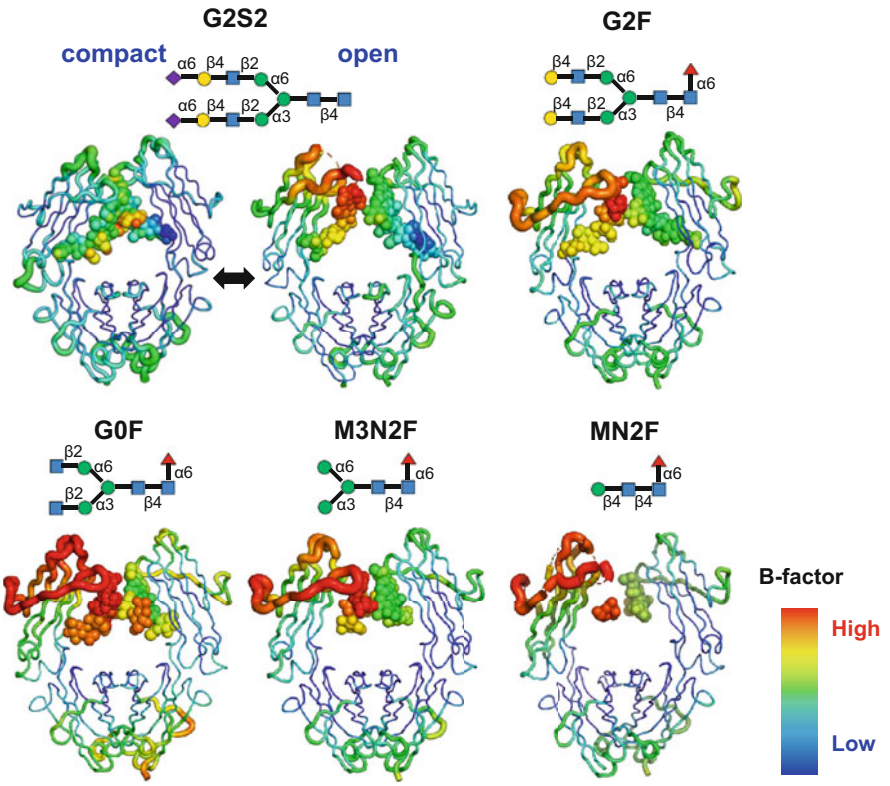

Fig. 15.4 Dynamic features of IgG-Fc glycoforms. High B-factors are indicated by red and thick tubes in a series of truncated glycoforms of IgG1-Fc (PDB ID codes: 5GSQ, 1H3V, 1H3X, 1H3U, and 1H3T). The shorter the oligosaccharides on the C_H2 domains the higher the B-factors, indicating increased mobility of the atoms in the C_H2 domains bearing short oligosaccharides. The molecular models were produced with PyMOL (The PyMOL Molecular Graphics System, Version 2.1.0, Schrödinger, LLC)

activities. By contrast, the horseshoe-shaped Fc opens further upon complex formation with an FcγR (Sondermann et al. 2000; Kiyoshi et al. 2015); it is presumed; therefore, that the different IgG-Fc glycans modulate the open conformation(s) of the IgG-Fc to afford optimal interactions/binding to effector molecules. This notion is supported by the crystal structures of the Fc glycoforms bearing sequentially truncated glycans ($(G2F)_2$, $(G0F)_2$, $(M3N2F)_2$ and $(MN2F)_2$, G: galactose; M: mannose; N: GlcNAc; F: fucose) in which the distance between the Pro329-Pro329 Cα residues are equated with the extent of openness; thus the distance between the Pro residues of the $(G2F)_2$ glycoform is measured as ~26.6 Å whereas for the $(MN2F)_2$ glycoform the distance is ~21.9 Å (Krapp et al. 2003) (Fig. 15.3b). Truncated IgG–Fc glycoforms showed progressive increases in the B-factors for the hinge proximal region of the C_H2 domains, as evidence of progressive structural disorder (Fig. 15.4) (Krapp et al. 2003). Progressive truncation of the terminal sugar

residues results in decreased binding affinity to FcγRIIb and C1q (Mimura et al. 2000, 2001), and FcγRIII (Yamaguchi et al. 2006); these effector ligands engage amino acid residues within the lower hinge and hinge proximal region of the C_H2 domain (Shields et al. 2001; Idusogie et al. 2000; Sarmay et al. 1992; Lund et al. 1990, 1991). It is interesting to note that subtle modulations of the IgG-Fc structure can result in profound impacts on effector ligand engagement, as determined ex vivo. Importantly, the FcγRIII receptor has been shown to bear five oligosaccharide moieties that, individually, modulate IgG-Fc engagement (Ferrara et al. 2011; Patel et al. 2018; Shibata-Koyama et al. 2009). Therefore, the glycan profiles of the IgG-Fc and each FcγR, in concert, can exert profound regulation of outcomes in vivo.

15.2.1 Fucosylation of IgG-Fc

The increased binding affinity to FcγRIII and mediation of ADCC for nonfucosylated G0/G1/G2 glycoforms in comparison with G0F/G1F/G2F glycoforms of IgG1 has been exploited to increase the efficacy of approved antibody therapeutics. Additionally, human IgG1 binds more strongly to NK cells homozygous for the FcγRIIIa-Val158 allotype than to those expressing FcγRIIIa-Phe158 allotype (Koene et al. 1997; Wu et al. 1997): nonfucosylated IgG exhibits higher binding affinity to the recombinant exo-domains of both FcγRIIIa-Val158 and FcγRIIIa-Phe158, 50-fold and 30-fold, respectively (Shields et al. 2002; Ferrara et al. 2006b). The enhanced FcγRIIIa binding of nonfucosylated Fc is dependent on the glycosylation status of FcγRIIIa, which has five potential glycosylation sites (Asn38, Asn45, Asn74, Asn162, and Asn169). Interestingly, the increased affinity for the nonfucosylated glycoform of IgG-Fc was negated when FcγRIIIa was not glycosylated at Asn162 (Ferrara et al. 2006b). The oligosaccharide attached at Asn162 is required for high-affinity binding to nonfucosylated Fc glycoforms while glycosylation of Asn45 has an inhibitory effect on the binding to nonfucosylated Fc (Shibata-Koyama et al. 2009); however, glycosylation at this site is required for expression of the protein in culture (Ferrara et al. 2011). In addition, individual glycoforms present at Asn162 also influence the interaction between the two components. The oligosaccharides of FcγRIIIa on NK cells from human donors are of complex- and oligomannose-type (Edberg and Kimberly 1997) whereas those of recombinant FcγRIIIa are of complex-type (Hayes et al. 2017). The affinity of FcγRIIIa bearing oligomannose-type oligosaccharides is ~twofold higher than FcγRIIIa with complex-type oligosaccharides (Ferrara et al. 2011). Increased affinity binding of nonfucosylated IgG is similarly observed for FcγRIIIb which is expressed on neutrophils and mediates degranulation and phagocytosis of antibody-opsonized targets (Subedi and Barb 2016; Dekkers et al. 2017).

The crystallographic studies of the complex between nonfucosylated IgG-Fc and glycosylated FcγRIIIa revealed unique interactions between the nonfucosylated oligosaccharide at Asn297 of IgG-Fc and the oligosaccharide at Asn162 of

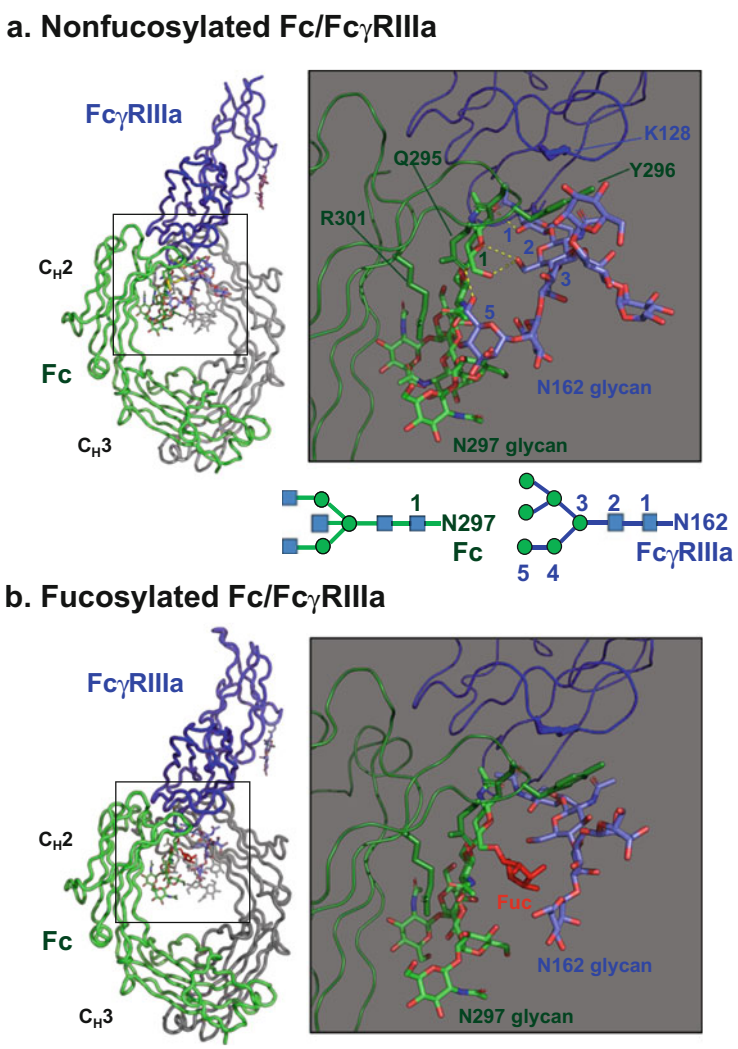

Fig. 15.5 Structures of nonfucosylated and fucosylated Fc fragments complexed with FcγRIIIa. Crystal structures of the complexes between nonfucosylated Fc and FcγRIIIa (PDB ID code: 3SGK) (**a**) and between fucosylated Fc and FcγRIIIa (PDB ID code: 3SGJ) (B) are shown (Ferrara et al. 2011). Hydrogen bonds between GlcNAc1 and GlcNAc2 of the Asn162 glycan of FcγRIIIa and GlcNAc1 of the nonfucosylated glycan of the Fc and between Man5 of FcγRIIIa and Gln295 of the Fc are observed as yellow dashed lines. Tyr296 of the Fc makes contacts with Man3 and Lys128 of FcγRIIIa. The fucose residue of the Fc glycan is shown in red (**b**). The molecular models were produced with PyMOL (The PyMOL Molecular Graphics System, Version 2.1.0, Schrödinger, LLC)

sFcγRIIIa (Ferrara et al. 2011; Mizushima et al. 2011) (Fig. 15.5a). Non-fucosylation of IgG-Fc increases carbohydrate–carbohydrate and carbohydrate–protein interactions between FcγRIIIa and IgG-Fc, thereby stabilizing complex formation. The crystal structure of the fucosylated Fc–glycosylated FcγRIIIa complex showed that the core fucose residue is oriented toward the secondary GlcNAc residue of the oligosaccharide at Asn162 of sFcγRIIIa, indicating steric inhibition of the glycosylated FcγRIIIa binding by Fc fucosylation (Fig. 15.5b). Thus, high-affinity IgG-Fc/FcγRIIIa binding requires an interaction of sugar residues attached at Asn162 with surface structures of the nonfucosylated IgG-Fc glycoform and due to the asymmetry of the IgG-Fc/FcγRIIIa interaction non-fucosylation of one heavy chain should be sufficient for tight binding.

Although most studies were conducted with IgG1 subclass proteins, increased ADCC was also demonstrated for nonfucosylated IgG3 and IgG4 antibodies; some activity also being observed for IgG2 (Niwa et al. 2005). Increased ADCC activity was also reported for a nonfucosylated glycoform of C_H1/C_L-deleted fusion protein and could, presumably, be extended to IgG-Fc fusion proteins (Natsume et al. 2005).

15.2.2 Galactosylation of IgG-Fc

Early studies of endogenous (polyclonal) serum-derived IgG showed it to be comprised of 22% G0(F), 40% G1(F), and 38% G2(F) (Jefferis et al. 1990); however, a similar analysis of monoclonal IgG, isolated from serum collected from patients with multiple myeloma showed each sample to express a restricted glycoform profile and highly variable proportions of each glycoform (Jefferis et al. 1990). A possible relevance of IgG-Fc galactosylation to function was first posited following the demonstration that IgG isolated from the sera of patients diagnosed with rheumatoid arthritis exhibited a deficit in galactosylation relative to that isolated from the sera of individuals in normal health; however, conflicting claims were reported mainly due to the fact that, at that time: (1) the role of fucosylation was not appreciated and, (2) that ~20% of serum IgG bears additional glycosylation site(s) within the Fab region (Jefferis 2017a). Subsequently, the glycoform profile of serum IgG has been shown to vary depending on age, sex, pregnancy, and disease (Parekh et al. 1985, 1988; Cheng et al. 2020; Ercan et al. 2012; Gudelj et al. 2018). The oligosaccharide profile of recombinant IgG produced in cell culture varies depending on the production platform employed and may be altered by precise manipulation of the composition of the culture medium, temperature, culture period, etc. (Raju and Jordan 2012).

Employing glycoengineered G0 and G2 IgG glycoforms of known fucosylation levels, it has been established that galactosylation of IgG-Fc has positive effects on C1q and FcγRIIIa binding and activation (Wada et al. 2019; Aoyama et al. 2019; Kurogochi et al. 2015; Dekkers et al. 2017; Thomann et al. 2016; Houde et al. 2010). Although the increase of FcγRIIIa binding by Fc galactosylation is subtle (~twofold) as compared with prominent enhancement by non-fucosylation of IgG-Fc, the

positive effect of galactosylation is independent of non-fucosylation (Houde et al. 2010; Thomann et al. 2016). In addition, Fc galactosylation is also shown to increase affinities to FcγRIIa/b (Subedi and Barb 2016; Thomann et al. 2015).

Although the molecular basis for the beneficial effects of galactosylation is not fully elucidated, the terminal galactose residue(s) contribute to the stability of IgG-Fc structure, as shown by an increase of the enthalpy for the unfolding of the C_H2 domains (Ghirlando et al. 1999; Wada et al. 2019) and lowered deuterium uptake in the hydrophobic surface of the C_H2 domain spanning Phe241 to Met252 (Aoyama et al. 2019). Furthermore, the crystal structure of the $(G2F)_2$ Fc glycoform shows the open conformation of the two C_H2 domains of the horseshoe-shaped Fc, which may favor FcγR binding (Fig. 15.3b) (Krapp et al. 2003; Sondermann et al. 2000).

Galactosylated IgG is increased in sera during pregnancy and preferentially transported to the fetus via the placenta (Kibe et al. 1996; Williams et al. 1995). While placental transport of maternal IgG is mediated by the neonatal Fc receptor (FcRn), numerous studies have suggested that noncanonical placental Fc receptors including FcγRIIb and FcγRIIIa expressed on placental endothelial cells and trophoblast cells, respectively, are involved in placental IgG transport (Martinez et al. 2019; Ishikawa et al. 2015; Wilcox et al. 2017; Jennewein et al. 2019).

15.2.3 Sialylation of IgG-Fc

The oligosaccharide of human serum IgG-Fc is hypo-sialylated at ~15% in α(2-6)-linkage, being mostly monosialylated on α(1-3)-arm and sparsely disialylated (Mimura et al. 2018; Arnold et al. 2006). The paucity of sialylation is presumed to reflect the intimate integration of the oligosaccharides within the IgG-Fc structure such that the steric/spatial requirements of the α(2-6)-sialyltransferase cannot be met (Jefferis 2017a). In fact, it is not easy to prepare homogeneous disialylated IgG by the in vitro glycoengineering approach using a combination of β(1-4)-galactosyltransferase and α(2-6)-sialyltransferase (Thomann et al. 2015).

Structural analyses of highly sialylated Fc revealed that the sialic acid residues are highly dynamic and free of strong interaction with the protein moiety of the Fc (Crispin et al. 2013; Barb et al. 2009, 2012; Ahmed et al. 2014; Chen et al. 2017). The terminal sialic acid on the α(1-6)-arm projects away from the protein surface in a solvent-exposed manner (Fig. 15.2b, shown in purple) while both the terminal sialic acid and the galactose residue on the α(1-3)-arm are not visible (PDB ID codes: 4Q6Y and 5GSQ).

The crystallographic studies revealed conformational heterogeneity in disialylated Fcs, which adopt open and compact conformations per asymmetric unit in the crystal (Fig. 15.4) (Ahmed et al. 2014; Chen et al. 2017). In the open conformer, the terminal sialic acid residue on the α(1–6)-arm interacts through water-mediated hydrogen bonds with the α-helix at the C_H2/C_H3 domain interface (Fig. 15.2b, shown in orange) while in the compact conformer the oligosaccharides

mutually interact and the sialic acid residue on the α(1-6)-arm is free of interaction with the C_H2/C_H3 domain. For nonfucosylated IgG, α(2-6)-sialylation slightly decreases FcγRIIIa binding and ADCC although these activities are several-fold higher than those of native IgG (Wada et al. 2019; Dekkers et al. 2017). On the other hand, for α(2-3) sialylated IgG the terminal α(2-3) sialic acid on the α(1-6)-arm is shown to weaken the interaction between the Fc oligosaccharide and the C_H2 domain possibly due to steric hindrance (Zhang et al. 2019; Kuhne et al. 2019), which is consistent with the reduced ADCC for α(2-3)-sialylated IgG (Scallon et al. 2007).

There are contradictory reports concerning biological consequences of α(2-6) sialylation of IgG-Fc. This relates to attempts to elucidate the mechanism(s) by which high doses of intravenous immunoglobulin (IVIG) mediate an anti-inflammatory activity in various human autoimmune/inflammatory diseases. In a mouse arthritis model a prophylactic use of α(2-6) sialylated IVIG was effective in the prevention of joint inflammation (Kaneko et al. 2006), and subsequently, the C-type lectin receptor SIGN-R1 in mice, or DC-SIGN in humans, was reported to engage sialylated Fc, which resulted in upregulated expression of FcγRIIb on macrophages, attenuating autoantibody-initiated inflammation (Anthony et al. 2011). However, it has been asserted that caution should be exercised when extrapolating from mouse models to humans, since the tissue distribution of SIGN-R1 and DC-SIGN differs. Other studies showed that the efficacy of IVIG to ameliorate mouse models of autoimmune diseases was independent of Fc sialylation or FcγRIIb (Leontyev et al. 2012; Guhr et al. 2011; Campbell et al. 2014; Othy et al. 2014; Yu et al. 2013) and that DC-SIGN does not interact with human IgG-Fc, regardless of glycosylation status (Yu et al. 2013; Temming et al. 2019). As usual for apparently contradictory scientific reports, both outcomes may be valid but critically dependent on precise experimental protocols and animal models (Schwab and Nimmerjahn 2014).

15.3 Impact of Glycosylation of mAbs and Fc-Fusion Proteins on Pharmacokinetics

The catabolic half-life of human IgG1, IgG2, and IgG4 is ~23 days, the longest of any serum protein, while for IgG3 it varies between allotypes and is ~7 or ~23 days in IgG3-Arg435 or IgG3-His435-expressing individuals, respectively (Stapleton et al. 2011; Braster et al. 2017; Vidarsson et al. 2014). IgG antibodies are protected from degradation in lysosomes through the FcRn recycling mechanism (Roopenian and Akilesh 2007). FcRn interacts with IgG at the C_H2/C_H3 interface with high affinity at an acidic pH (<6.5) but not at a physiological pH (Burmeister et al. 1994). Although FcRn binding to IgG is presumed to be independent of Fc glycosylation, longer retention times have been noted by FcRn affinity chromatography for galactosylated and sialylated IgG glycoforms than more truncated glycoforms

during elution with a linear pH gradient from pH 5.5 to 8.8 (Cymer et al. 2017; Wada et al. 2019); however, an association between differential retention patterns of IgG glycoforms in an FcRn column and pharmacokinetics remains unknown. Other receptors that are known to bind and clear proteins with specific glycans include the asialoglycoprotein receptor that binds to terminal galactose residues of N-glycans (Ashwell and Harford 1982; Stockert 1995) and the mannose receptor that recognizes terminal mannose or GlcNAc sugars (Lee et al. 2002).

Oligomannose-type glycoforms are found at low proportions when produced from Chinese hamster ovary (CHO) and murine cells (Mimura et al. 2009; Goetze et al. 2011; Zhang et al. 2016). It should be noted that while oligomannose-type IgG glycoforms exhibit higher ADCC activity than fucosylated complex-type IgG glycoforms, shorter half-lives have been demonstrated for the former than the latter (Kanda et al. 2007b; Liu et al. 2011; Alessandri et al. 2012; Liu 2015). When therapeutic IgG1 or IgG2 antibody was administered in human subjects, the relative abundance of IgG glycoforms with terminal galactose or GlcNAc remained constant during 34 days after injection while oligomannose-type IgG glycoforms were selectively cleared more rapidly at lower intravenous doses (Goetze et al. 2011). There might be an association between the number of oligomannose glycans on IgG-Fc and in vivo clearance. In the investigation of the impact of IgG-Fc oligomannose glycan pairing on antibody clearance, it was demonstrated that IgG1 and IgG2 mAbs with both symmetrical and asymmetrical pairings exhibited similarly fast clearance in humans, independently of the extent of mannosylation (Liu and Flynn 2016). Thus, the presence of oligomannose-type glycoforms may compromise the efficacy of antibody therapeutics through enhanced clearance and/or possible immunogenicity elicited by uptake of immune complexes via the mannose receptor on macrophages/dendritic cells and the activation of the mannan-binding lectin pathway (Jefferis 2017b; Arnold et al. 2006).

The Fab is also glycosylated in 15–20% of polyclonal human IgG and the Fab oligosaccharides can be of highly galactosylated and sialylated complex-type (Mimura et al. 2007; Holland et al. 2006) or of oligomannose-type, depending on the location of the glycosylation site in the V_H region (Gala and Morrison 2004; Wright et al. 1991; Radcliffe et al. 2007). However, it is important to emphasize that IgG-Fab glycosylation has not been shown to compromise clearance rates, at least in mouse models (Huang et al. 2006).

On the other hand, it has been reported that the clearance rate of Fc-fusion proteins can be influenced by the fusion partner and its attached oligosaccharides (Liu 2015). Fc-fusion proteins may also have N-glycosylation sites in the fusion partner and the attached oligosaccharides can be highly galactosylated and sialylated, in contrast to those of the Fc portion. Although the catabolic half-lives of some Fc-fusion proteins are shorter than intact IgG molecules, possibly reflecting reduced affinity of Fc-fusion proteins to FcRn (Suzuki et al. 2010), the quantities of total sialic acids in the fusion partner of etanercept are positively correlated with a catabolic half-life in rats (Liu 2015).

15.4 Glycosylation of Total IgG and Antigen-Specific IgG in Autoimmunity

An association of increased levels of agalactosylated IgG in serum with disease activity in rheumatoid arthritis has raised the question of whether the agalactosylated glycoforms are causative (proinflammatory) or a bystander phenomenon (Parekh et al. 1985; Ercan et al. 2010; Stambuk et al. 2020). In fact, galactosylation of IgG has been shown to increase affinities for FcγRIIIa and C1q and results in increased ADCC and CDC activities (Dekkers et al. 2017), respectively, while agalactosylated IgG-containing immune complexes may activate the lectin pathway of complement via mannan-binding lectin (Malhotra et al. 1995; Banda et al. 2008). Vidarsson and coworkers have recently proposed differential effects of certain IgG-Fc glycoforms on disease activity of autoimmunity between antigen-specific IgG (auto- or alloantibodies) and nonspecific, total IgG (irrelevant bulk antibodies) (Dekkers et al. 2018). Galactosylated (G2) glycoforms of total IgG antibodies are presumed to occupy FcγRs, raising activation thresholds and preventing antigen-specific IgG from engagement with FcγRs including FcγRIIIa/b during remission. Upon relapse, however, agalactosylated (G0) glycoforms of total IgG are dissociated from FcγRs, lowering the threshold and allowing antigen-specific antibodies to provoke inflammation (Fig. 15.6).

Oligosaccharide profiles of antigen-specific IgG (e.g., ACPA and anti-red blood cell autoantibodies) and total IgG have been analyzed and compared before and after relapse in patients with autoimmune diseases (Sonneveld et al. 2017, 2018; Rombouts et al. 2015; Kemna et al. 2017). Notably, in Granulomatosis with polyangiitis glycosylation of total IgG differed from that of proteinase 3-anti-neutrophil cytoplasmic antibody (PR3-ANCA) IgG1 (Wuhrer et al. 2015; Kemna et al. 2017). For example, it has been reported that during relapse the oligosaccharide profiles of total IgG exhibited significantly decreased galactosylation/bisection and increased fucosylation while those of PR3-ANCA showed decreased galactosylation/fucosylation. On the other hand, in non-relapsing patients, the oligosaccharide profiles of total IgG were not significantly altered while those of PR3-ANCA showed decreased galactosylation/fucosylation and increased bisection (Kemna et al. 2017). Notably, a comparison of the oligosaccharide profiles of total IgG between relapsing and non-relapsing patients reveals higher levels of galactosylation, bisection, and non-fucosylation for the latter. These observations are consistent with the possible inhibitory activity of galactosylated species of total IgG, which may account for the association of low galactosylation levels of total IgG with severity in autoimmunity. Even if an increase in affinity of IgG for FcγRs by galactosylation is subtle, synergistic effect of galactosylation and non-fucosylation on affinity to FcγRs may overcome high-avidity of immune complexes. Although the proportion of plasma IgG bearing at least one nonfucosylated oligosaccharide is estimated to be as low as ~10% (Kapur et al. 2014), oligosaccharide analysis of IgG1 isolated from FcγRIIIa on human NK cells revealed that >50% of the oligosaccharides were nonfucosylated (Patel et al. 2019). This suggests that nonfucosylated IgG

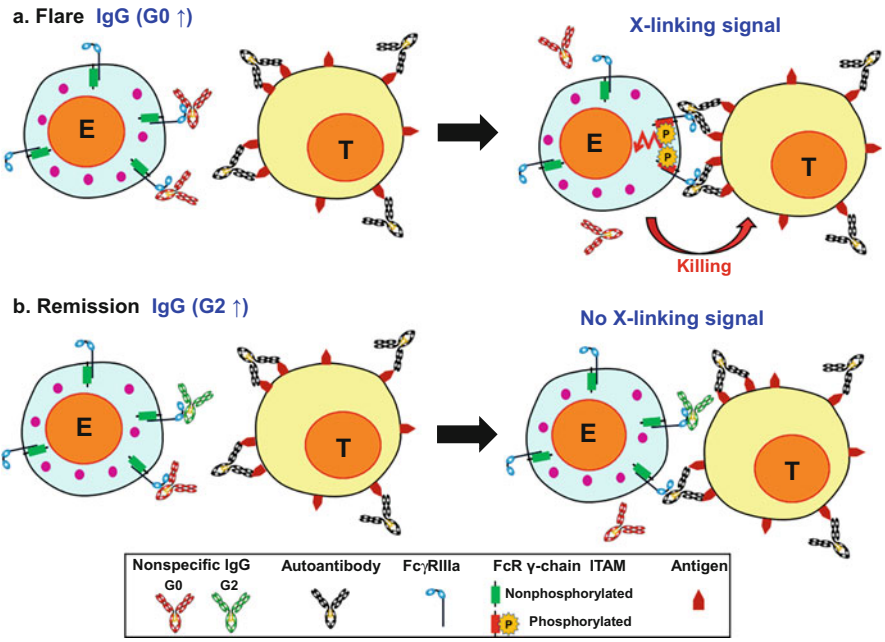

Fig. 15.6 Hypothetical role for glycoforms of plasma IgG in blockade of FcγRs on immune effector cells. (**a**) Nonspecific agalactosylated IgGs (G0) cannot compete with high-avidity autoantibody-antigen complexes on a target cell for FcγR binding, resulting in cross-linking FcγRs in effector cells during a flare. (**b**) During remission, nonspecific galactosylated IgGs (G2) occupy FcγRs and suppress the generation of cross-linking signals via FcγRs. E, effector cell. T, target cell

substantially occupies FcγRIIIa/b on circulating NK cells and neutrophils, potentially suppressing the recognition of autoantibody-opsonized target cells by NK cells and neutrophils. In addition, galactosylation can increase FcγRIIa binding, therefore, this provides a clue to enhancement of the anti-inflammatory activity of total IgG (i.e., IVIG) via glycoengineering.

IVIG is a therapeutic preparation of polyclonal IgG, derived from pooled plasma of thousands of healthy donors, used for the treatment of autoimmune/inflammatory diseases, including Kawasaki Disease and Guillain–Barré syndrome. The anti-inflammatory activity of IVIG is shown to reside in the Fc from a clinical study on the treatment of immune thrombocytopenic purpura with the Fc fragments of IVIG (Debre et al. 1993), and multiple mechanisms of action have been proposed for the efficacy of IVIG, including FcγR blockade and FcγRIIb upregulation. As anti-inflammatory activity of IVIG requires Fc glycosylation and large quantities (1–3 g of IgG/kg of body weight), it is postulated that the active constituent of IVIG can be certain IgG-Fc glycoforms such as sialylated glycoforms (Nimmerjahn and Ravetch 2008). The precise mechanism of action of IVIG in autoimmune diseases remains elusive and the issue as to whether sialylated glycoforms exert anti-inflammatory effect remains an open question. As stated above, it is postulated

that in autoimmune diseases, including rheumatoid arthritis, the alterations in glycosylation of total IgG that modulate ADCC and ADCP play a key role in the disease activity.

15.5 Glycoengineering

Glycoproteins are comprised of heterogeneous glycoforms, and separating them into individual glycoforms has been challenging due to the attachment of diverse glycan structures as well as variations in glycosylation site occupancy. Glycoengineering approaches have been developed to generate or enrich desirable glycoforms to enhance the biological properties of glycoprotein therapeutics with maximized safety and efficacy. Cell glycoengineering approaches are based on overexpression and knockout/knockdown of certain glycoprocessing enzymes in mammalian (e.g., hamster, murine, rat, and human) (Yang et al. 2015; Goh et al. 2014; Yamane-Ohnuki et al. 2004) and non-mammalian host cells (e.g., insect, plant, and yeast) (Li et al. 2006; Le et al. 2016; Strasser et al. 2008; Mabashi-Asazuma et al. 2014), knockout of genes of enzymes involved in sugar-nucleotide biosynthesis and loss-of-function mutations in the Golgi sugar-nucleotide transporter genes (Kanda et al. 2007a; Kelly et al. 2018).

For the last decade, a variety of host cell engineering approaches have been directed toward the preparation of fucose-deficient IgG to enhance the affinity for FcγRIIIa and ADCC activity. Among common approaches are the modifications of the fucosylation pathway in CHO cell lines, including (1) knockout/knockdown of α1,6-fucosyltransferase (FUT8) gene using sequential homologous recombination (Potelligent® technology, Kyowa Hakko Kirin) (Yamane-Ohnuki et al. 2004), co-expression of β1,4-*N*-acetylglucosaminyltransferase-III (GnT-III) and β-mannosidase-II (Man-II) (GlycoMAb® technology, Roche) that localize the GnT-III to the early Golgi compartment, resulting in increased addition of bisecting GlcNAc and increased inhibition of the addition of fucose (Ferrara et al. 2006a); (2) disruption of the de novo GDP-fucose biosynthetic pathway in CHO cells (GlymaxX® technology, ProBioGen); (3) inhibitors of cellular protein fucosylation (fluorinated fucose analogs) (Okeley et al. 2013; Dekkers et al. 2016). Several glycoengineered, therapeutic IgG antibodies lacking core fucose or with low fucose content have entered the clinic, including mogamulizumab, benralizumab, obinutuzumab, etc.

15.5.1 Chemoenzymatic Glycoengineering

The in vitro chemoenzymatic glycan remodeling approach by combined use of endo-β-*N*-acetylglucosaminidase (ENGase), glycosynthase, and sugar oxazoline has a capability to prepare potentially any desired homogeneous glycoform of

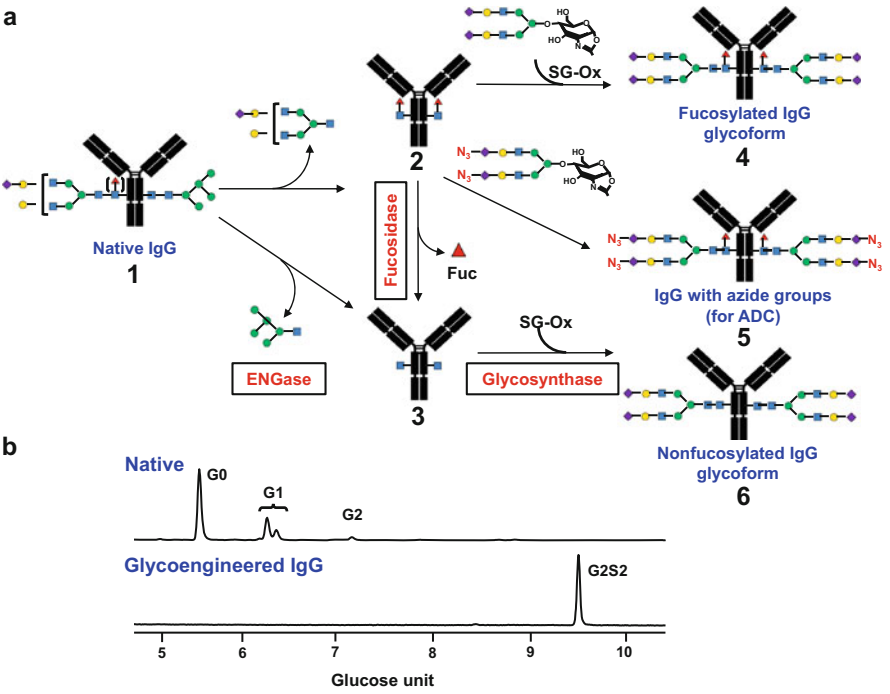

Fig. 15.7 Endoglycosidase-catalyzed glycan remodeling of IgG-Fc. (**a**) Schematic representation of the chemoenzymatic glycoengineering method. (**b**) UPLC-HILIC profiles of native (top) and G2S2 oligosaccharide (bottom) released from IgG1 mAb mogamulizumab glycoengineered with EndoM-N175Q (Tokyo Chemical Industry, Japan). *ENGase* endo-β-*N*-acetylglucosaminidase, *SG-Ox* sialoglycan oxazoline, *ADC* antibody-drug conjugate

IgG-Fc. Details on the approach are provided by a recent review by Wang et al. (2019). The approach involves cleavage of N-glycan(s) at the chitobiose core leaving the innermost GlcNAc or Fuc-GlcNAc with ENGase, together with fucosidase digestion where necessary, and transfer of a preassembled glycan to the innermost GlcNAc of the acceptor protein with ENGase-based glycosynthase (Fig. 15.7). The approach utilizes highly active glycan oxazolines, the mimics of the transition state, as donor substrates (Kobayashi et al. 1996), and transglycosylation with the synthetic glycan oxazoline proceeds in both a stereo- and regiospecific manner (Li et al. 2005). Various structures of glycan oxazoline are synthesized by endoglycosidase digestion of intact or exoglycosidase-digested sialylglycopeptide (SGP) from egg yolk and condensation with 2-chloro-1,3-dimethyl-imidazolinium chloride (DMC) in the presence of triethylamine (Sun et al. 2014; Noguchi et al. 2009). Homogeneous IgG glycoforms prepared by this method include fully sialylated diantennary and triantennary complex-type, hybrid-type, and oligomannose-type glycoforms (Lin et al. 2015; Li et al. 2016).

This method is based on the discovery of the transglycosylation activity of various ENGases, including EndoA from *Arthrobacter protophormiae* (Takegawa

et al. 1995, 1997), EndoM from *Mucor hiemalis* (Yamamoto et al. 1994; Fujita et al. 2004), and EndoD from *Streptococcus pneumoniae* (Fan et al. 2012; Muramatsu et al. 2001) of the glycoside hydrolase (GH)-85 family; and Endo-S from *Streptococcus pyogenes* (Huang et al. 2012), EndoS2 from *Streptococcus pyogenes* NZ131 of serotype M49 (Li et al. 2016), and EndoF3 from *Elizabethkingia meningoseptica* (Giddens et al. 2016) of the GH18 family. To date, numerous mutants of ENGases have been generated as glycosynthases that abolish the hydrolytic activity on the transglycosylation products and improve the transglycosylation efficiency although different ENGases have distinct substrate specificity and limitations. EndoM acts on both the complex-type and oligomannose-type glycans whereas EndoA or EndoS acts on the oligomannose-type or the complex-type, respectively.

The first ENGase-catalyzed remodeling of IgG glycoforms was performed by deglycosylation of yeast-expressed human IgG1-Fc with EndoH and transglycosylation with EndoA of synthetic glycan-oxazolines including $Man_3GlcNAc$-oxazoline to the innermost GlcNAc residue of IgG-Fc (Wei et al. 2008). Furthermore, EndoA was found to efficiently catalyze transglycosylation to IgG-Fc of truncated glycan-oxazolines including bisecting GlcNAc-containing $Man_3GlcNAc$-oxazoline, which showed a higher affinity of the bisected $Man_3GlcNAc_2$-bearing Fc for FcγRIIIa, relative to the counterpart Fc glycoform without bisecting GlcNAc (Zou et al. 2011). In addition, glycosynthase mutants from EndoD (EndoD-N332Q) and EndoM (EndoM-N175Q) were reported to exhibit the transglycosylation activity to IgG and ribonuclease A (Fan et al. 2012; Huang et al. 2009); however, the former does not transfer full-length complex-type glycan to the Fc while the latter does not use fucosylated Fc as a substrate, which limits the applicability of the chemoenzymatic approach. The discovery of EndoS mutants D233A and D233Q represents a breakthrough of this method as these glycosynthase mutants catalyze the transfer of full-length complex-type glycan-oxazoline to both nonfucosylated and fucosylated GlcNAc-IgG with high efficiency (Huang et al. 2012). Furthermore, the D184M and D184Q mutants of EndoS2 have broader substrate specificity than EndoS-D233Q (Li et al. 2016), acting on the complex-type, hybrid-type, and oligomannose-type glycans. EndoF3-D165A and D165Q can transfer both di- and triantennary oligosaccharides to intact IgG-Fc (Giddens et al. 2016). According to recent protocols, transglycosylation of glycan oxazoline to IgG (~5 mg/ml) can be achieved by EndoS-D233Q or EndoS2-D184M within 4 h at 30 °C at a glycan oxazoline/IgG molar ratio of 40 (Tang et al. 2017; Li et al. 2018).

The structural integrity of transglycosylated IgG products is presumed to be maintained. Comparison of crystal structures of glycoengineered Fc glycoform and native Fc revealed no major difference in the conformation between the transferred and native oligosaccharides and in the orientation of hydrophobic side chains adjacent to each oligosaccharide (Fig. 15.2) (Ahmed et al. 2014; Chen et al. 2017). With regard to the functionality, rituximab and trastuzumab with the Fc glycans defucosylated via the chemoenzymatic glycoengineering exhibited enhanced FcγRIIIa binding and ADCC, as observed for nonfucosylated IgG variants produced in FUT8-knockout CHO host cells (Li et al. 2018; Lin et al. 2015; Chen et al. 2017;

Kurogochi et al. 2015; Huang et al. 2012; Liu et al. 2018). It should be noted that, currently, only this chemoenzymatic glycoengineering approach allows for the preparation of homogeneous disialylated IgG-Fc at high efficiency, which is superior to conventional approaches that combine βGal4T1 and ST6Gal1 treatment (Thomann et al. 2015). Recently, homogeneous IgG glycoforms prepared by this method have been utilized to investigate the stability and functionality of individual IgG glycoforms (Lin et al. 2015; Ahmed et al. 2014; Wada et al. 2019; Li et al. 2017). Another breakthrough is the discovery of α1,6-fucosidases that can remove core fucose from the innermost GlcNAc of IgG-Fc (Tang et al. 2017; Tsai et al. 2017). This chemoenzymatic glycoengineering approach has also been exploited to ADCs because of its robust, stable, and site-specific conjugation.

15.5.2 Antibody-Drug Conjugates Via Glycosylation

The Fc oligosaccharide is exploited as an attractive reaction site to prepare ADCs. Most of ADCs approved for clinical use are based on the conjugation of cytotoxic drugs to Lys (trastuzumab emtansine (Kadcyla®), inotuzumab ozogamicin (Besponsa®)) and Cys residues (brentuximab vedotin (Adcetris®)), resulting in a variable distribution of DAR. It has been demonstrated that random conjugation has a negative impact on therapeutic efficacy. To circumvent the structural heterogeneity, various methods have been developed that utilize the Fc oligosaccharide as ADC conjugation site (Toftevall et al. 2020) (Fig. 15.8). Zhou and coworkers have developed a glycosylation site-specific method consisting of enzymatic remodeling of the Fc oligosaccharide with a combination of β4GalT1 with ST6Gal1, periodate oxidation of the terminal sialic acids to generate aldehyde groups and conjugation of aminooxy functionalized cytotoxic agents via oxime ligation (Zhou et al. 2014). This method converts diantennary complex-type oligosaccharides to monosialylated forms (>94%) and incorporates ~1.6 cytotoxic agents per antibody molecule. However, periodate treatment can also oxidize Met residues, and the DAR is generally low. van Geel and coworkers at SynAffix have developed the GlycoConnect™ technology which consists of oligosaccharide release with EndoS2 and the transfer of azido group-containing GalNAc (*N*-azidoacetylgalactosamine) to the primary GlclNAc residue by β4GalT Y289L, which serves as a chemical handle for conjugation with a cytotoxic agent (Fig. 15.8a) (van Geel et al. 2015). The applicability of this technology is shown by using different isotypes and linker–drug combinations. Huang and coworkers have recently reported an alternative approach via EndoS-catalyzed Fc oligosaccharide remodeling in which native Fc oligosaccharides are replaced with N_3-modified sialylated complex-type oligosaccharides as a chemical handle, by deglycosylation with ENGase and reglycosylation with glycosynthase (e.g., EndoS-D233Q) (Figs. 15.7a and 15.8b) (Tang et al. 2017). The approaches using the Fc oligosaccharides have some advantages, including the controlled DAR as 2.0, the stability of

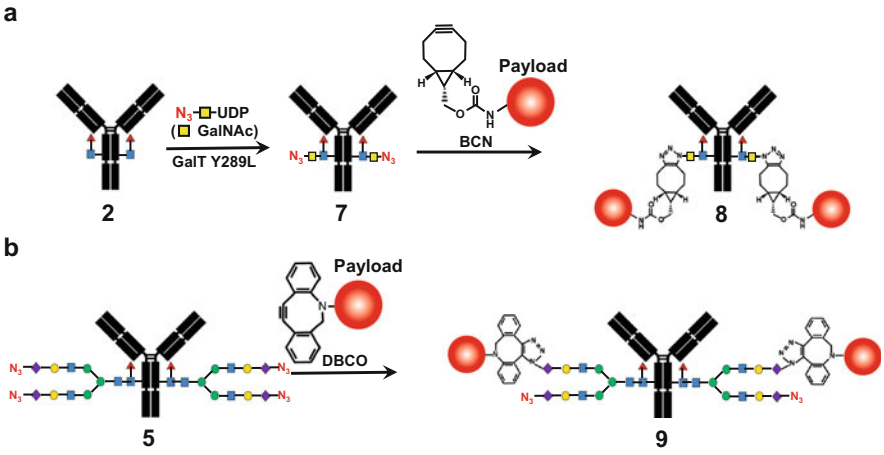

Fig. 15.8 Chemoenzymatic syntheses of glycosylation site-specific antibody-drug conjugates (ADCs). (**a**) Transfer of azido-functionalized GalNAc to the core GlcNAc of IgG (Structure **2** in Fig. 15.7) with GalT (Y289L) and site-specific conjugation of bicyclo[6.1.0]nonyne (BCN)-tagged drug payload to the azide-group by click chemistry (van Geel et al. 2015). (**b**) Site-specific conjugation of dibenzoazacyclooctyne (DBCO)-tagged drug payload to the azido-functionalized N-glycans of IgG (Structure **5** in Fig. 15.7) via a copper-free "click" reaction, yielding ADC. Synthesis of DBCO-tagged cytotoxic agent is described in Tang et al. (2017)

the conjugates in the circulation, and homogeneity of the predefined ADC structure (Tang et al. 2016, 2017; Wang et al. 2019).

IgM antibody is also considered as a promising platform for ADC due to the presence of 51 oligosaccharides in a pentameric IgM antibody including J-chain (Moh et al. 2019). By using ST6Gal1 and azide-functionalized CMP-sialic acid, conjugation of a drug candidate having dibenzoazacyclooctyne (DBCO) group to azide-sialic labeled oligosaccharides on IgM is achieved by copper-free click chemistry, with a DAR of 8–10.

15.5.3 Glycoengineering of Antibodies for Treatment of Cancers, Inflammation, and Infectious Diseases

Glycoengineering has been applied to optimize the effector functions of therapeutic IgG antibodies for the treatment of cancers, inflammatory, and infectious diseases. Numerous glycoengineered antibodies, including fucose-deficient or aglycosylated IgG antibodies, have entered the clinic or have been evaluated under clinical trials.

15.5.3.1 Cancers

As of 2020, ~20 mAbs in clinical development have been glycoengineered to deplete core fucose for cancer treatment (Kaplon et al. 2020; Pereira et al. 2018). The first approved nonfucosylated therapeutic antibody is humanized anti-CC chemokine receptor 4 (CCR4) IgG1 antibody mogamulizumab (Poteligeo®) for treatment of CCR4-positive adult T-cell leukemia or relapsed peripheral T-cell lymphoma (Ishida et al. 2012). Other nonfucosylated IgG antibodies include humanized anti-CD20 IgG1 obinutuzumab (Gazyva®, approved in 2013, USA) and primatized anti-EGFR IgG1 imgatuzumab (Gonzalez-Nicolini et al. 2016). Mogamulizumab is produced in FUT8$^{-/-}$ CHO cells (Potelligent® technology) and completely devoid of core fucose (Fig. 15.1b). Obinutuzumab with a low fucose content is produced in CHO-K1 cells engineered to overexpress GnT-III and Golgi α-mannosidase II (GlycoMab® technology) and exhibits superior antitumor activities to the reference antibody rituximab (Sehn et al. 2012, 2015). Fucose depletion of the licensed antitumor therapeutic IgG antibodies rituximab and trastuzumab has been shown to enhance ADCC activities (Iida et al. 2006; Junttila et al. 2010). In fact, nonfucosylated anti-CD20 antibody obinutuzumab combined with cytotoxic agent chlorambucil has shown better outcome than rituximab in patients with chronic lymphocytic leukemia despite infusion-related reactions and neutropenia (Goede et al. 2014).

The efficacy of anticancer therapeutic antibodies is influenced by FcγRIIIa-Val158Phe polymorphism, with patients homozygous for FcγRIIIa-Val158 showing higher clinical responses compared to FcγRIIIa-Phe158 carriers (Overdijk et al. 2014). Nonfucosylated glycoforms of anticancer therapeutic antibodies have some benefits, with regard to (1) the ability to elicit potent ADCC in individuals irrespective of the FcγRIIIa-Val158Phe allotype (Niwa et al. 2004), (2) the ability to mediate ADCC in the presence of large excess of plasma IgG (Nechansky et al. 2007), (3) lower doses compared with fucosylated mAbs (e.g., only 1 mg/kg for mogamulizumab) (Iida et al. 2006), and (4) the ability to mediate monocyte and macrophage phagocytic and cytotoxic activities (Herter et al. 2014). Obinutuzumab-induced ADCC is not negatively influenced by KIR/HLA interactions (Terszowski et al. 2014). Furthermore, the enhanced efficacy of obinutuzumab is explained by the ability to induce higher IFN-γ secretion from NK cells than rituximab upon interaction with the antibody-opsonized tumor cells irrespective of FcγRIIIa-Val158Phe polymorphism (Capuano et al. 2017). It is suggested that, besides short-term cytotoxic properties, obinutuzumab-experienced NK cells promote dendritic cell maturation and long-lasting T cell responses via increased IFNγ secretion.

15.5.3.2 Autoimmune and Inflammatory Diseases

Glycoengineered mAb therapeutics for autoimmune/inflammatory diseases currently approved or under clinical evaluation also have low fucose contents.

Nonfucosylated humanized anti-IL-5Rα IgG1 benralizumab (Fasenra®) produced in FUT8$^{-/-}$ CHO cells (Potelligent® technology) has been approved for the treatment of severe, uncontrolled asthma, with eosinophilic phenotype (Wang et al. 2017a). Complete depletion of eosinophils and their early progenitors in the bone marrow has been reported in eosinophilic asthmatic patients who received benralizumab (Sehmi et al. 2018), and its clinical efficacy was comparable to the anti-IL-5 antibody mepolizumab (Bourdin et al. 2018). Nonfucosylated humanized anti-CD19 inebilizumab (Uplizna®) has received its first global approval in 2020 for treatment of neuromyelitis optica spectrum disorder, an autoimmune, demyelinating disease of the central nervous system, which is indicated for adult patients who are seropositive for anti-aquaporin-4 autoantibodies (Frampton 2020). This antibody therapeutic is also under clinical evaluation for kidney transplant desensitization, myasthenia gravis, and IgG4-related disease through depletion of CD19-expressing B cells by ADCC.

Roledumab (LFB-R593) is a human IgG1 anti-rhesus (Rh)D mAb with a low fucose content produced from rat YB2/0 cells by EMABling® technology (Yver et al. 2012), and its safety and efficacy are being evaluated in the Phase 2/3 NCT02287896 study for the prevention of feto-maternal alloimmunization in RhD-negative women, as a substitute for human polyclonal anti-RhD antibodies. Interestingly, plasma anti-RhD IgG antibodies (RhIG) from hyperimmunized healthy anti-D donors are found to have lowered Fc fucosylation (47% for females) compared with normal IgG (93%) (Kapur et al. 2015). Fc fucosylation levels of RhIG from manufacturers are variably lowered (56%–91%), which may inversely correlate with the efficiency of clearance of D+ red blood cells by FcγRs on macrophages and NK cells from maternal circulation. Although the precise mechanism of action of RhIG is not known, high-affinity binding of nonfucosylated RhIG to FcγRIIIa and enhanced ADCC may be related to successful prevention of maternal immunization with RhD-positive red blood cells.

15.5.3.3 Infection

Neutralizing mAbs specific for viruses including influenza A and respiratory syncytial virus (RSV) are known to confer potent protection against infection. Humanized anti-RSV IgG1 mAb palivizumab (Synagis®) is recommended for prophylaxis of high-risk neonates during bronchiolitis seasons; however, the therapeutic efficacy of the antibody for the treatment of RSV infection has not been demonstrated (Alansari et al. 2019). Interestingly, a glycovariant of palivizumab produced in a genetically engineered plant host bearing mostly nonfucosylated, nongalactosylated glycans showed increased FcγRIIIa binding and decreased RSV titers in the lungs of cotton rats, compared with the licensed fucosylated palivizumab (Hiatt et al. 2014). Thus, the induction of ADCC is important for successful protection against viral infection, which is also noted for influenza A (He et al. 2016; Jegaskanda et al. 2019). Anti-hemagglutinin stalk domain antibodies are efficient at induction of ADCC, in contrast to anti-neuraminidase antibodies, but the latter has an additive effect on

ADCC with the former. For antibodies against HIV, dengue virus, and SARS-CoV-2, the oligosaccharide profiles of the specific antibodies from the infected subjects are characterized by elevated levels of nonfucosylated IgG glycoforms. For HIV, this was observed for those who had a longer disease-free survival which may correlate with antibody-mediated cellular viral inhibition (Ackerman et al. 2013); however, for dengue virus and SARS-CoV-2, it may cause antibody-dependent enhancement of infection (Wang et al. 2017b; Weber and Oxenius 2014; Bournazos et al. 2020; Chakraborty et al. 2021; Larsen et al. 2020). It remains unknown what factors determine the outcome in the presence of nonfucosylated antibodies against these infections. Nonetheless, glycoengineering to deplete core fucose of IgG for enhanced ADCC activity may provide an opportunity to improve the treatment of viral infection.

15.6 Conclusion

The improved clinical efficacy of nonfucosylated IgGs through enhanced FcγRIIIa binding and ADCC activity has boosted the development of the glycoengineered mAbs for the treatment of cancers and chronic inflammatory diseases. A protective role of ADCC against infectious diseases has also been noted including RSV, dengue virus, influenza virus, and HIV. A nonfucosylated variant of the broadly neutralizing human IgG1 anti-HIV mAb b12 exhibits greater in vitro ADCC activity against HIV-infected cells compared to fucosylated b12 (Moldt et al. 2012); however, no enhanced protection with nonfucosylated b12 was observed against a vaginal simian-human immunodeficiency virus challenge in rhesus macaques, compared to fucosylated b12. These findings suggest the requirement of FcγR-mediated activities other than FcγRIIIa-mediated ADCC for protection against viral infection.

The long-standing question of whether agalactosylated IgG is proinflammatory whereas sialylated and/or galactosylated IgG are anti-inflammatory in autoimmune diseases has been approached by observation of differential Fc oligosaccharide profiles between total IgG and antigen-specific IgG antibodies. Galactosylation of total IgG may raise the threshold for FcγR activation of immune effector cells through improved binding affinity for FcγRs, and the opposite may be true for agalactosylation of total IgG. Additionally, it seems likely that circulating nonfucosylated IgGs dominantly occupy FcγRIIIa/b, presumably raising the threshold for activation of NK cells and neutrophils. Increased levels of fucosylated IgG have been reported for total IgG in patients with rheumatoid arthritis (Gornik et al. 1999) and Granulomatosis with polyangiitis (Kemna et al. 2017), which may reflect a decrease of circulating nonfucosylated IgG as a result of binding to FcγRIIIa/b on immune cells that expand during a flare. If it is the case, nonfucosylated, galactosylated IVIG may serve as promising anti-inflammatory therapeutics for autoimmune diseases, inhibiting FcγRs-mediated immune cell activation through FcγRIIIa/b blockade (Mimura et al. 2022).

The introduction of chemoenzymatic glycoengineering method employing glycosynthase mutants allows remodeling of IgG glycoforms at high efficiency and flexibility. This approach has several advantages over conventional in vitro glycoengineering approaches, with respect to a reaction time and the capabilities for defucosylation, sialylation, bisection, and branching. It should be noted that the longer the reaction time, the higher the risk of spontaneous degradations (e.g., deamidation and oxidation, see Chap. 1). Transfer of pre-defined oligosaccharides to IgG-Fc *en bloc* can mostly be achieved within a few hours, maintaining homogeneity of a glycoproteoform. Thus, this novel chemoenzymatic glycoengineering approach has been employed in numerous studies on the structure/function of IgG-Fc glycoforms (Wada et al. 2019; Aoyama et al. 2019; Li et al. 2017; Ahmed et al. 2014; Chen et al. 2017) and opens a new avenue to glycoform remodeling for therapeutic purposes. Overall, the oligosaccharides of IgG-Fc are the fertile ground for the development of next-generation antibody therapeutics with improved efficacy.

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Compliance with Ethical Standards

Conflict of Interest Yusuke Mimura, Radka Saldova, Yuka Mimura-Kimura, Pauline M Rudd, and Roy Jefferis declare that they have no conflict of interest.

Ethical Approval This chapter does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 16
Glycosylation of Plant-Produced Immunoglobulins

Kathrin Göritzer and Richard Strasser

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Abstract Many economically important protein-based therapeutics like monoclonal antibodies are glycosylated. Due to the recognized importance of this type of posttranslational modification, glycoengineering of expression systems to obtain highly active and homogenous therapeutics is an emerging field. Although most of the monoclonal antibodies on the market are still produced in mammalian expression platforms, plants are emerging as an alternative cost-effective and scalable production platform that allows precise engineering of glycosylation to produce targeted human glycoforms at large homogeneity. Apart from producing more effective antibodies, pure glycoforms are required in efforts to link biological functions to specific glycan structures. Much is already known about the role of IgG1 glycosylation and this antibody class is the dominant recombinant format that has been expressed in plants. By contrast, little attention has been paid to the glycoengineering of recombinant IgG subtypes and the other four classes of human immunoglobulins (IgA, IgD, IgE, and IgM). Except for IgD, all these

K. Göritzer
St. George's University of London, London, UK

R. Strasser (✉)
University of Natural Resources and Life Sciences Vienna, Vienna, Austria
e-mail: richard.strasser@boku.ac.at

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antibody classes have been expressed in plants and the glycosylation has been analyzed in a site-specific manner. Here, we summarize the current data on glycosylation of plant-produced monoclonal antibodies and discuss the findings in the light of known functions for these glycans.

Keywords Antibody · Glycan · Glycoengineering · Glycoprotein · *Nicotiana benthamiana* · Plant biotechnology · Recombinant protein

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AG	Arabinogalactan
AGP	Arabinogalactan proteins
ALG	Asn-linked glycosylation
CHO	Chinese hamster ovary
CNX	Calnexin
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated-9
CRT	Calreticulin
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
Fc	Fragment crystallizable
FUT	Fucosyltransferase
GALT	Galactosyltransferase
GMII	Golgi mannosidase II
GnGn	$GlcNAc_2Man_3GlcNAc_2$ N-glycan
GnGnXF	$GlcNAc_2XylFucMan_3GlcNAc_2$ N-glycan
GnT	*N*-acetylglucosaminyltransferase
HEK	Human embryonic kidney
HEXO	*N*-acetylhexosaminidase
Hyp	Hydroxyproline
Ig	Immunoglobulin
JC	Joining chain
MMXF	$Man_3XylFucGlcNAc_2$
MNS	Mannosidase
MUC1	Mucin 1
OST	Oligosaccharyltransferase
P4H	Prolyl-4-hydroxylase
pIgR	Polymeric immunoglobulin receptor
SC	Secretory component
SIgA	Secretory IgA
ST	Sialyltransferase

STT3	Staurosporine and temperature sensitive 3
XylT	Xylosyltransferase

16.1 Introduction to N-Glycan Processing in Plants

N-Glycosylation of secretory proteins is initiated by the en bloc transfer of a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) precursor in the lumen of the endoplasmic reticulum (ER). The assembly of the lipid-linked oligosaccharide precursor involves multiple Asn-linked glycosylation (ALG) enzymes that are all conserved in plants (Strasser 2016). The $Glc_3Man_9GlcNAc_2$ moiety is transferred by the oligosaccharyltransferase (OST) complex to asparagine residues in the sequence Asn-X-Ser/Thr (X can be any amino acid except proline) of newly synthesized polypeptides. In budding yeast, mammals, and plants, OST is a multimeric membrane-bound protein complex consisting of one catalytically active subunit (STT3) and several different non-catalytic subunits that mediate interactions with the translocation channel and ribosome or might be required for glycosylation of specific sites (Shrimal and Gilmore 2019). Mammals harbor two different OST complexes. While the STT3A complex interacts with the translocon and mediates co-translational glycosylation, the STT3B complex catalyzes posttranslational glycosylation of proteins and glycosylates sites that have been skipped by the STT3A complex. Plants have also two catalytic subunits, termed STT3A and STT3B (Koiwa et al. 2003) that likely form two distinct heteromeric OST complexes (Niu et al. 2020). However, the function of individual OST subunits appears different in plants (Farid et al. 2013; Castilho et al. 2018) and our current understanding of the role of the STT3A and STT3B complexes in N-glycosylation of plant proteins is still limited (Jeong et al. 2018).

Once the oligosaccharide has been transferred by the OST complex, the N-glycan is subjected to stepwise processing. Removal of the terminal α1,2-linked glucose by α-glucosidase I and subsequent removal of the first α1,3-linked glucose by α-glucosidase II result in a glycan structure that can be recognized by the lectin chaperones calnexin (CNX) and calreticulin (CRT) that promote protein folding (Strasser 2018). In this ER-quality control process, misfolded glycoproteins are subjected to several rounds of interaction with CNX/CRT and monitoring of their folding status. Proteins that have acquired their native conformation are released from the CNX/CRT cycle and allowed to exit the ER to downstream compartments. Terminally misfolded proteins are recognized by a poorly understood process and directed towards ER-associated degradation (ERAD) to prevent the accumulation or secretion of potentially harmful proteins. The basic biological functions of the glycan-dependent ER quality control process and clearance mechanism of aberrant glycoproteins are conserved in plants.

Correctly folded and assembled secretory glycoproteins leave the ER and transit through the Golgi apparatus where they encounter multiple glycosidases and

glycosyltransferases that process oligomannosidic N-glycans to complex N-glycans (Strasser 2016). While in budding yeast and mammals, the first trimming reaction is catalyzed by an ER-resident α-mannosidase, MNS3 the corresponding plant enzyme is primarily located in the *cis*-Golgi (Schoberer et al. 2019). MNS3 catalyzes the trimming of a single α1,2-linked mannose from the middle branch of the $Man_9GlcNAc_2$ N-glycan to form $Man_8GlcNAc_2$. Subsequently, three additional mannose residues are cleaved off by Golgi α-mannosidase I. MNS1 and MNS2 are two functionally redundant Golgi α-mannosidases in *Arabidopsis thaliana* (Liebminger et al. 2009). The resulting $Man_5GlcNAc_2$ structure is used by β1,2-*N*-acetylglucosaminyltransferase I (GnTI) to initiate hybrid and complex N-glycan formation (von Schaewen et al. 1993; Strasser et al. 1999). The transfer of the GlcNAc residue to the α1,3-linked mannose by GnTI is required for further N-glycan modifications in the Golgi. In the next processing steps, Golgi α-mannosidase II (GMII) removes the α1,6- and α-1,3-linked mannose residues and β1,2-*N*-acetylglucosaminyltransferase II (GnTII) attaches a single GlcNAc to the α1,6-linked mannose to generate the complex N-glycan $GlcNAc_2Man_3GlcNAc_2$ (GnGn) (Fig. 16.1). Until this step, the processing reactions are conserved between mammals and plants. Subsequently, a β1,2-linked xylose and a core α1,3-linked fucose are attached to GnGn to generate $GlcNAc_2XylFucMan_3GlcNAc_2$ (GnGnXF) the predominant complex N-glycan found on glycoproteins in plants (Wilson et al. 2001; Léonard et al. 2004; Strasser et al. 2004). Of note, the substrate specificities and the overlapping Golgi localization of the corresponding enzymes allow an alternative order of processing from $GlcNAcMan_5GlcNAc_2$ to GnGnXF with the β1,2-xylosyltransferase (XylT) activity preceding trimming by GMII (Strasser 2016). Further modifications of complex N-glycans are catalyzed by β1,3-galactosyltransferase 1 (GALT1) and α1,4-fucosyltransferase (FUT13). The two *trans*-Golgi resident enzymes generate the Lewis-a carbohydrate epitope [Fucα1,4 (Galβ1,3)GlcNAc-R] that is ubiquitously found in plants, but occurs only on a very limited number of plant glycoproteins (Fitchette-Lainé et al. 1997; Wilson et al. 2001; Strasser et al. 2007). Truncated $Man_3XylFucGlcNAc_2$ (MMXF) N-glycans are generated from GnGnXF by removal of terminal GlcNAc residues. This reaction is catalyzed either by the vacuolar β-*N*-acetylhexosaminidase 1 (HEXO1) or by an apoplast located HEXO3 that is the major contributor to the formation of truncated N-glycans on secreted glycoproteins (Liebminger et al. 2011; Shin et al. 2017).

Apart from the formation of Lewis-a structures, no further complex N-glycan modifications have been described. Plants lack *N*-acetylglucosaminyltransferases for the formation of tri- or tetra-antennary N-glycans and the attachment of a bisecting GlcNAc. Common mammalian complex N-glycan modifications that are found on immunoglobulins such as core α1,6-fucose and terminal β1,4-galactose have not been described (Strasser et al. 2009). Moreover, plants lack the biosynthesis pathway for CMP-sialic acid, a Golgi CMP-sialic acid transporter as well as α2,3- and α2,6-sialyltransferases (ST) that catalyze the transfer of CMP-Neu5Ac to complex N-glycans in the Golgi (Zeleny et al. 2006; Castilho et al. 2010). Due to the absence of these mammalian-type complex N-glycan modifications, the N-glycan heterogeneity on plant-produced glycoproteins is clearly reduced which is an enormous

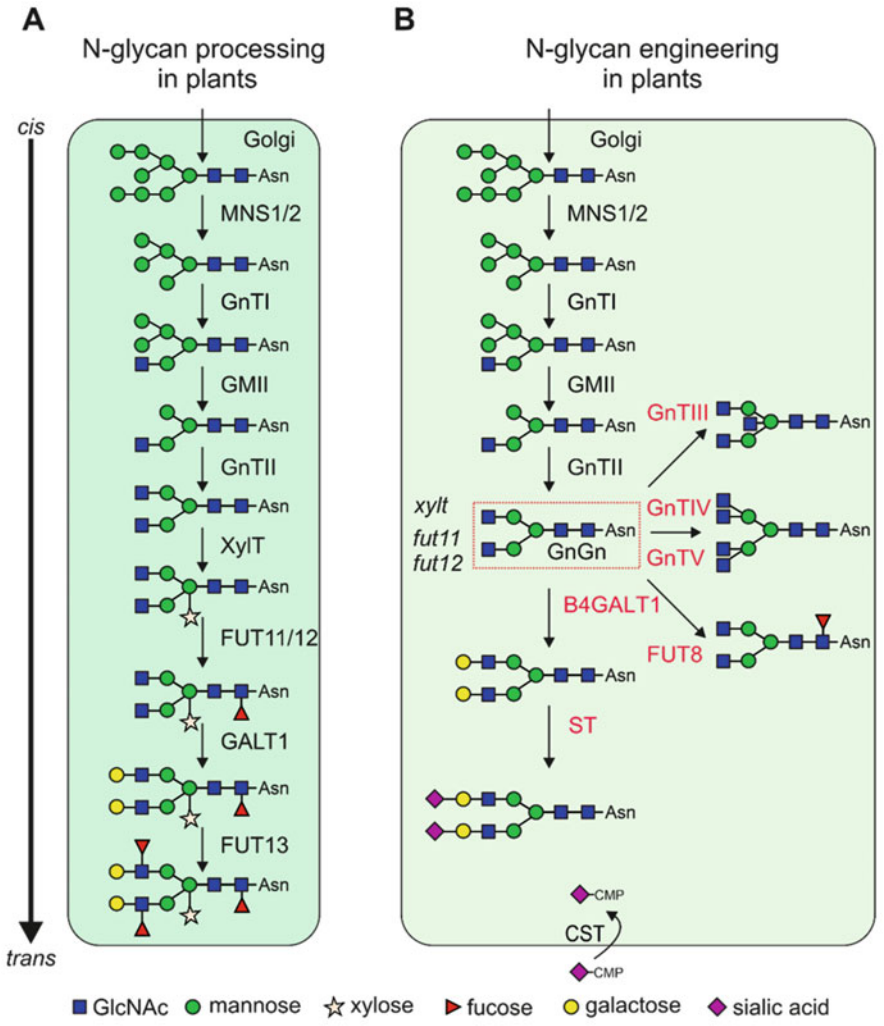

Fig. 16.1 (**a**) Schematic representation of complex N-glycan processing in the plant Golgi apparatus. Golgi-α-mannosidase I (MNS1/2), *N*-acetylglucosaminyltransferase I (GnTI), Golgi-α-mannosidase II (GMII), *N*-acetylglucosaminyltransferase II (GnTII), β1,2-xylosyltransferase (XylT), core α1,3-fucosyltransferase (FUT11/12), β1,3-galactosyltransferase (GALT1), and α1,4-fucosyltransferase (FUT13) are indicated. (**b**) N-glycan engineering approaches to produce defined homogenous complex N-glycans on plant-produced recombinant antibodies: the generation of *xylt*, *fut11 fut12* knockouts results in the formation of the GnGn structure which can serve as acceptor substrate for *N*-acetylglucosaminyltransferase III (GnTIII), IV (GnTIV), and V (GnTV), core α1,6-fucosyltransferase (FUT8), β1,4-galactosyltransferase (B4GALT1), and α2,6-sialyltransferases (ST). Sialylation in plants requires the co-expression of the Golgi CMP-sialic acid transporter (CST) and proteins for CMP-sialic acid biosynthesis (not shown)

advantage for approaches aiming at the generation of defined homogenous N-glycans for different applications or glycan structure function studies (Schoberer and Strasser 2018) (Fig. 16.1).

16.2 Introduction to O-Glycan Biosynthesis in Plants

In mammals, O-glycan biosynthesis occurs in a stepwise fashion involving the sequential transfer of single monosaccharide residues to secreted and membrane-bound proteins. During mucin-type O-glycan biosynthesis, which is the most common O-linked glycan in humans, polypeptide *N*-acetylgalactosaminyltransferases catalyze the transfer of an *N*-acetylgalactosamine (GalNAc) residue from the nucleotide sugar UDP-GalNAc to hydroxyl side chains of Ser/Thr to generate GalNAcα1-*O*-Ser/Thr (Tn antigen) (Bennett et al. 2012). This initial step in mucin-type O-glycan biosynthesis can be carried out by one of 20 different polypeptide GalNAc-transferases in the Golgi apparatus of mammalian cells. After the initial glycosylation reaction, multiple monosaccharides are attached to the Golgi in a stepwise manner yielding elongated and branched O-glycan core structures (Tran and Ten Hagen 2013). The most common extension is catalyzed by the core 1 β1,3-galactosyltransferase (T-synthase or C1GalT1), which adds galactose in a β1,3-linkage to generate the core 1 O-glycan structure Galβ1–3GalNAcα1-*O*-Ser/Thr (also known as T antigen). O-Glycan structures can be further modified with the addition of sugars such as galactose, GlcNAc, fucose, and terminal sialic acid. These elongated and branched mucin-type O-glycans are typically found on mammalian glycoproteins.

A similar mucin-type O-glycan biosynthesis pathway does not exist in plants (Strasser 2012). However, specific proline residues are converted by prolyl-4-hydroxylases (P4Hs) to hydroxyproline (Hyp) followed by a glycosylation reaction. Two major types of O-glycans are attached to glycoproteins with Hyp residues. Unbranched chains composed of up to five arabinose (Ara) residues are added to clusters of Hyp residues in proteins such as extensins, whereas complex arabinogalactans (AGs) are attached to clustered noncontiguous Hyp residues on arabinogalactan proteins (AGPs) (Ellis et al. 2010). The O-glycosylation of AGPs is initiated by a set of Hyp-galactosyltransferases that add a single galactose to a Hyp residue in the Golgi of plants. The AGP glycan structures are not well characterized but include incorporation of multiple galactose residues and additional modifications with arabinose, xylose, fucose, or glucuronic acid. On extensins, a single galactose can be attached to a Ser residue next to a Hyp repeat that is not further modified or elongated with other sugar residues.

16.3 Engineering of N-Glycan Processing Pathways in Plants

Initial attempts to engineer the N-glycan processing pathway in plants aimed to prevent the formation of complex or truncated N-glycans with β1,2-xylose and core α1,3-fucose. Both N-glycan modifications have been associated with an increased risk for immunogenicity and adverse allergic reactions in humans (Bardor et al. 2003; Jin et al. 2008; Paulus et al. 2011). The potential immunogenicity of the plant-specific N-glycans has been discussed extensively in the context of molecular farming, and there is still an ongoing debate whether β1,2-xylose and core α1,3-fucose modifications have an adverse effect when present on recombinant therapeutic proteins (Grabowski et al. 2014; Ward et al. 2014; Piron et al. 2015; Shaaltiel and Tekoah 2016; Rup et al. 2017). Furthermore, unwanted regulatory concerns make their elimination desirable.

Pioneering work in *A. thaliana* demonstrated that plants tolerate the complete removal of β1,2-xylose and core α1,3-fucose residues without any adverse effects on plant growth or development (Strasser et al. 2004). Based on this study, gene silencing approaches were successfully applied to almost completely remove these N-glycan modifications in the aquatic plant *Lemna minor* and in *Nicotiana benthamiana* (Cox et al. 2006; Strasser et al. 2008). *N. benthamiana* is currently used by academic groups and companies worldwide as a transient expression system for monoclonal antibodies, Fc-fusion proteins, virus-like particles, and antigens used for therapy, prophylaxis, and diagnostics (Stoger et al. 2014; Lomonossoff and D'Aoust 2016; Margolin et al. 2020; Sainsbury 2020). Multiplex CRISPR/Cas9 genome editing was recently used to generate *N. benthamiana* deficient in plant-specific core α1,3-fucosyltransferase and β1,2-xylosyltransferase activities (Jansing et al. 2019). Consistent with previous findings for *A. thaliana* (Strasser et al. 2004), no obvious phenotype was described for this multiple knockout lines highlighting that *N. benthamiana* plants tolerate the removal of plant-specific complex N-glycans very well. Anti-HIV IgG antibodies produced in the ΔXT/FT knockdown line (Strasser et al. 2008) or in the recently described knockout line (Jansing et al. 2019) displayed primarily GnGn N-glycans (Fig. 16.1). This human-type N-glycan structure is the preferred base for engineering glycan extensions and introduction of mammalian-type complex N-glycan modifications (Montero-Morales and Steinkellner 2018). GnGn N-glycans can serve as acceptor substrates for the attachment of β1,4-linked galactose (Strasser et al. 2009), branching (Castilho et al. 2011b; Nagels et al. 2011), bisecting GlcNAc (Castilho et al. 2015), core α1,6-fucosylation (Castilho et al. 2011a) or the formation of immunomodulatory helminth N-glycans carrying Galβ1–4(Fucα1–3)GlcNAc (Lewis X), or GalNAcβ1–4GlcNAc (LDN) structures (Wilbers et al. 2017). The formation of complex N-glycans with β1,4-galactose in plants paves the way for subsequent sialylation which has been achieved by transient or stable expression of the mammalian sialylation machinery (Castilho et al. 2010; Kallolimath et al. 2016). Using these approaches, complex N-glycan

branches were capped with single α2,3- or α2,6-linked sialic acid residues or further extended with α2,8-linked polysialic acid (Kallolimath et al. 2016).

Other glycoengineering approaches are intended to eliminate interfering complex N-glycan modifications. The Lewis-a epitope formation interferes with β1,4-galactosylation and potentially presents an immunogenic epitope when highly abundant on recombinant therapeutic proteins. Lewis-a structures have, for example, been detected on recombinant human erythropoietin (EPO) produced in *N. benthamiana* and *Physcomitrella patens* (Weise et al. 2007; Castilho et al. 2011b). Knockout of the GALT1 orthologue in *P. patens* prevented the formation of the Lewis-a epitope on recombinant EPO (Parsons et al. 2012). Transient knockdown of HEXO3 in *N. benthamiana* enriched the amount of GnGn-containing N-glycans on recombinant glycoproteins (Shin et al. 2017) and depletion of a specific β-galactosidase from the apoplast of *N. benthamiana* prevented the removal of β1,4-galactose from recombinant glycoproteins (Kriechbaum et al. 2020). Complete knockout of the endogenous plant genes coding for these glycosyl hydrolases will further improve the *N. benthamiana* expression system resulting in the formation of recombinant glycoproteins with highly homogeneous glycans.

Besides differences in N-glycan processing, some recombinant proteins expressed in plants are underglycosylated (Van Droogenbroeck et al. 2007; Hamorsky et al. 2015; Castilho et al. 2018; Göritzer et al. 2019; Montero-Morales et al. 2019; Stelter et al. 2020). The reduced N-glycosylation efficiency is caused by yet unknown differences in the function of the plant OST complex. For some recombinant proteins including antibodies, the underglycosylation of N-glycosylation sites can be overcome by co-expression of a single subunit OST from *Leishmania major* (LmSTT3D) (Castilho et al. 2018; Montero-Morales et al. 2019; Göritzer et al. 2020).

16.4 Engineering of O-Glycosylation Biosynthesis Pathways in Plants

Despite the huge differences between mammalian and plant-type O-glycans, comparatively little attempts have been directed toward the production of human-type O-glycans in plants. The analysis of plant-produced recombinant proteins carrying mucin-type O-glycosylation sites revealed the presence of Hyp as well as several pentose residues corresponding to unbranched arabinose chains found on plant extensins (Karnoup et al. 2005; Pinkhasov et al. 2011; Castilho et al. 2012; Yang et al. 2012; Dicker et al. 2016; Göritzer et al. 2017). Hyp residues are not found on human proteins such as IgA1 or EPO. The presence of the arabinose chain may cause adverse effects and bears the risk of an unwanted immune response against plant-produced therapeutic proteins. Therefore, one aim of plant O-glycan engineering approaches is the elimination of specific P4H activities to prevent Hyp formation and subsequent plant-specific glycosylation. In *P. patens*, knockout of a single P4H

completely abolished the production of Hyp on recombinant EPO (Parsons et al. 2013). Plants like *A. thaliana* or *N. benthamiana* have numerous potential P4H candidates that could be involved in the hydroxylation of proline on recombinant proteins (Velasquez et al. 2011). Consequently, the removal of the plant-specific modification will likely require the knockout of several P4H genes coding for enzymes with similar substrate specificities.

In addition to the removal of the unwanted native O-glycosylation repertoire of plants, other engineering strategies aimed to introduce mucin-type O-glycosylation. Human polypeptide GalNAc-transferase 2 has, for example, been transiently expressed in *N. benthamiana* to initiate O-GalNAc formation on different recombinant glycoproteins including peptides derived from human mucin 1 (MUC1), EPO-Fc, or IgA1 (Pinkhasov et al. 2011; Castilho et al. 2012; Yang et al. 2012; Dicker et al. 2016). On the single O-glycosylation site of human EPO-Fc, the core 1 structure could be generated by expression of the *Drosophila melanogaster* core 1 β1,3-galactosyltransferase. Co-expression of the mammalian CMP-sialic acid biosynthesis pathway, the CMP-sialic acid transporter, and the corresponding sialyltransferases led to the production of IgA1 or EPO-Fc with sialylated mucin-type O-glycans (Castilho et al. 2012; Dicker et al. 2016). For the generation of defined O-glycan structures on recombinant proteins, the absence of an endogenous mucin-type O-glycan biosynthesis pathway is of great advantage as it allows the stepwise modification of O-glycans with only the desired monosaccharides.

16.5 Glycosylation of Plant-Produced IgGs

In wild-type plant-produced IgGs, the Fc-resident GnGn N-glycan (Fig. 16.1) is commonly modified with β1,2-xylose and core α1,3-fucose residues to produce GnGnXF which is not present in mammals. In glycoengineered *N. benthamiana*, IgG with humanized complex-type GnGn N-glycans as major glycoforms were produced (Strasser et al. 2008; Jansing et al. 2019). Besides the conserved N-glycosylation sites on the Fc portion (Fig. 16.2), additional carbohydrate chains can be linked to the hypervariable regions of IgG. For instance, up to 25% of IgG molecules isolated from the serum of healthy individuals as well as several therapeutic monoclonal antibodies like cetuximab have been reported to carry N-glycans on their variable domains which exhibit site-specific differences compared to the Fc-resident N-glycan on the same molecule. While the Fc N-glycan of such antibodies is less modified, the N-glycan in the variable region is more exposed and displays extensive processing (Teh et al. 2014). Similarly, such IgG1 antibodies produced in plants were shown to carry up to 30% α1,3-fucose residues in the Fab resident N-glycan revealing a leaky knockdown in the ΔXT/FT plants (Castilho et al. 2015).

On some plant-produced IgGs low amounts of truncated structures have been detected (Strasser et al. 2008; Stelter et al. 2020). Depending on the IgG idiotype expressed, also small amounts of not fully processed oligomannosidic structures can

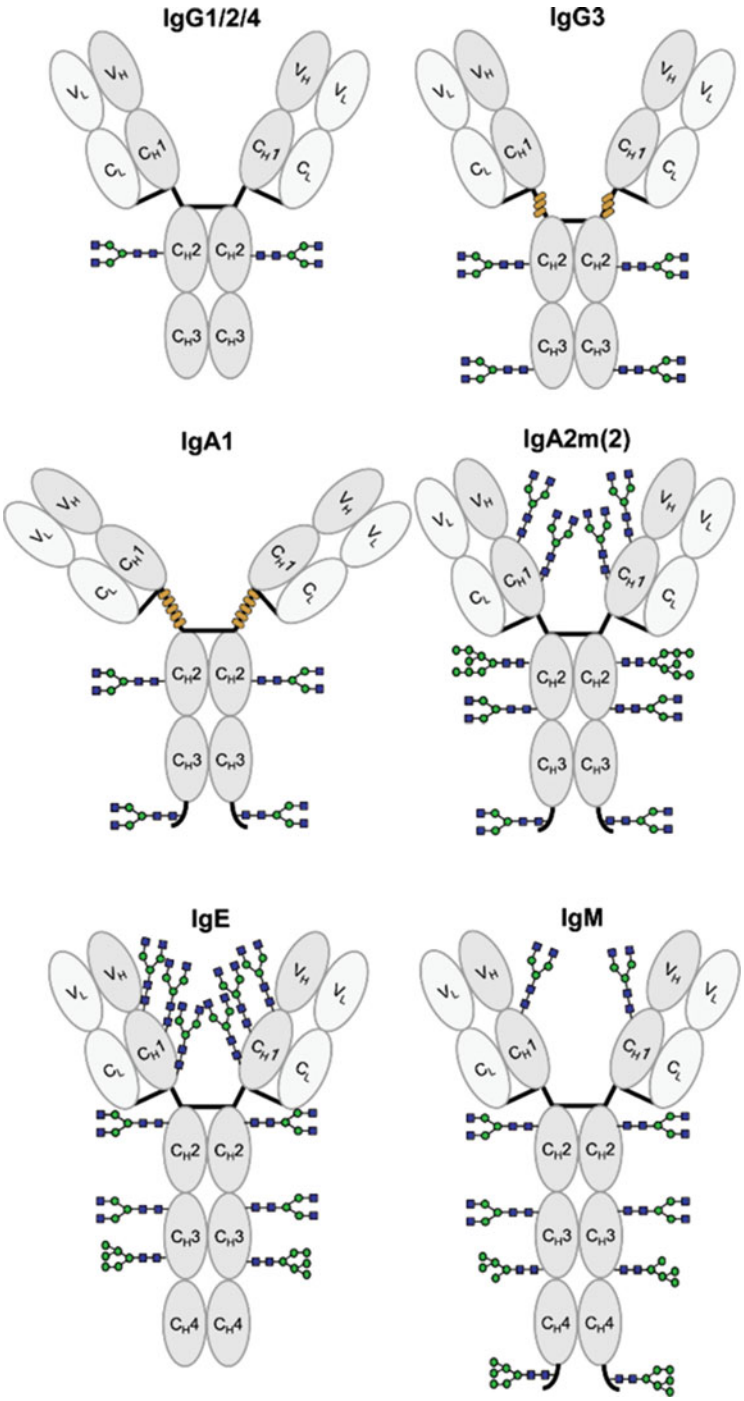

Fig. 16.2 Schematic illustration of the structure and glycosylation sites of IgGs, IgAs, IgE, and IgM. The light chain is colored in light gray and the heavy chain in dark gray. N-glycans found in

occur resulting from different secretion efficiency to the apoplast due to ER retention of potentially incompletely folded IgGs (Westerhof et al. 2014). An issue that has only been tackled recently are the differences in N-glycosylation occupancy of plant- and mammalian-produced glycoproteins. While the single N-glycosylation site present in the human IgG Fc region is almost 100% glycosylated when expressed in mammalian cells, 10–30% of plant-produced IgG is underglycosylated at this site due to yet unknown features of the plant OST complex (Castilho et al. 2018; Stelter et al. 2020). However, transient expression of the single-subunit OST from *L. major* successfully increases the N-glycan occupancy on the IgG Fc site. Using these different strategies, plants can produce IgGs with very little microheterogeneity carrying a homogenous glycosylation profile with mostly GnGn N-glycans.

Glycoengineering of IgG has focused mainly on the elimination of core fucose from the N-glycan in the Fc region of the heavy chain as major contributions to antibody activities have been assigned to that N-glycan residue (Umaña et al. 1999; Shinkawa et al. 2003; Yamane-Ohnuki et al. 2004; Junttila et al. 2010). However, co-expression of the responsible mammalian core α1,6-fucosyltransferase in glycoengineered ΔXT/FT plants facilitated the generation of IgGs with and without fucose while retaining an otherwise identical N-glycosylation profile (Forthal et al. 2010; Castilho et al. 2011a). This led to a series of studies of plant-produced IgG showing that the absence of fucose increases the affinity for FcγRIII receptor binding and improved antibody-dependent cellular cytotoxicity (ADCC) on natural killer cells (Jez et al. 2012; Loos and Steinkellner 2012; Qiu et al. 2014; Marusic et al. 2018; Stelter et al. 2020). A similar glycosylation-dependent mechanism has an impact on antibody-dependent cellular phagocytosis (ADCP) by macrophages and influences the receptor-mediated effector function of virus-neutralizing antibodies (Forthal et al. 2010; Lai et al. 2014; Hayes et al. 2017). Furthermore, it has been suggested that α1,6-linked fucose could contribute to antibody-dependent enhancement (ADE) of infection and therefore plant-produced IgGs with GnGn could be safer and more efficacious antibody-based therapeutics against dengue virus and other ADE-prone viral diseases (Dent et al. 2016; Hurtado et al. 2020). The success of afucosylated IgG antibodies produced in plants is highlighted with the case of ZMapp, an antibody cocktail for treatment of Ebola virus infections, which was used during the Ebola outbreak in 2014/2015 (Qiu et al. 2014). Core fucose-free monoclonal antibody 13F6 which is one of the ZMAPP components displayed clearly enhanced potency against Ebola virus compared to 13F6 variants with core fucose (Castilho et al. 2011a).

Fig. 16.2 (continued) the constant domains of the different antibody classes are indicated with symbols that are drawn according to the nomenclature from the Consortium for Functional Glycomics (http://www.functionalglycomics.org/). For each site, the predominant N-glycan structure (complex GnGn or oligomannosidic) found on ΔXT/FT *N. benthamiana* produced recombinant antibody is indicated. Potential O-glycosylation sites are marked in the hinge region of IgG3 and IgA1 (orange ellipse)

Capping of both branches (>80%) of the IgG Fc glycan (Strasser et al. 2009; Stelter et al. 2020) with β1,4-galactosylated structures could be achieved by targeting human β1,4-galactosyltransferase (B4GALT1) to a late Golgi compartment in *N. benthamiana* ΔXT/FT (Strasser et al. 2009; Castilho et al. 2011a; Jez et al. 2012). This is an improvement compared to CHO cell-produced IgG that frequently carries galactose residues only on one branch. Employing these glycoengineering approaches in plants it could be shown that β1,4-galactosylation can, although not improving interaction with Fcγ receptors, enhance neutralization activity of two anti-HIV antibodies (Strasser et al. 2009; Stelter et al. 2020).

The final and most complex step of human complex N-glycan processing is terminal sialylation. These negatively charged residues at the nonreducing end of N-glycans reduce protein turnover by preventing the exposure of galactose, GlcNAc, or mannose to lectin receptors like the asialoglycoprotein receptor (Ashwell and Morell 1974). In addition, there is also long-standing evidence that IgG molecules can have anti-inflammatory activity in autoimmune diseases and recent studies indicate that this activity is associated with the presence of sialic acid (Kaneko et al. 2006; Raju and Lang 2014; Wang and Ravetch 2019). The synthesis of sialylated N-glycans in plants involves the coordinated co-expression of several mammalian proteins acting in different subcellular compartments at different stages of the N-glycosylation pathway (Castilho et al. 2010). While the *in planta* sialylation works quite well for proteins like EPO, IgG sialylation is only possible in the presence of a core fucose residue (Castilho et al. 2015; Kallolimath et al. 2020).

The plant-based production of IgG1 to IgG4 subtypes has been reported recently and all of them display a quite similar N-glycan profile when expressed in glycoengineered *N. benthamiana* (Kallolimath et al. 2020). IgG3 has a second N-glycosylation site in the CH3 domain (Fig. 16.2) and an extended hinge region that is very likely modified with Hyp and plant-specific O-glycans. When produced in *N. benthamiana*, IgG3 displayed degradation products of the heavy chain. Whether the cleavage takes place in the extended hinge region remains to be shown. Modification of the hinge region with human mucin-type O-glycans might be a valuable strategy to reduce the proteolytic vulnerability of the hinge region of recombinant IgG3 produced in plants. Altogether, glycoengineering in plant-based systems provides a reliable platform to generate human IgG antibodies with a controlled glycosylation pattern.

16.6 Glycosylation of Plant-Produced IgAs

IgAs are increasingly gaining attention as possible biopharmaceuticals for treatment of infectious diseases and cancer, especially in mucosal settings due to their unique structural and functional properties. The two IgA isotypes (IgA1 and IgA2) carry two to five N-glycosylation sites on the α-(heavy) chain. In addition, the IgA1 hinge region is elongated and modified with up to six O-linked glycans (Yoo and Morrison 2005). In serum, IgA occurs mostly as its monomeric structural unit, however, it can

be further assembled into dimers through incorporation of the joining chain (JC), a small polypeptide with a single biantennary complex N-glycan, which along with the IgA tailpiece N-glycan contributes to correct dimer formation (Atkin et al. 1996; Yoo et al. 1999; Göritzer et al. 2020). Newly synthesized dimeric IgA can associate with the pIgR receptor that is expressed as integral membrane protein on the basolateral side of epithelial cells lining mucosal surfaces, after which it is transported across the epithelium and released into the lumen. At the luminal side, pIgR is cleaved and a part referred to as secretory component (SC) remains attached thereby forming SIgA (Johansen et al. 2001; Mostov et al. 1984). The secretory component is a hydrophilic and highly glycosylated polypeptide carrying seven N-glycosylation sites, which protect SIgA from degradation and can interact with various host cell receptors and pathogens (Brandtzaeg 2013).

The N- and O-glycans attached to IgA in circulation are very heterogenous and their function is often not well understood. Therefore, generating recombinant monomeric and multimeric IgA variants bearing well-defined glycans is challenging but desired to study their contribution to IgA function. Furthermore, aberrant glycosylation such as galactose-deficient IgA1 O-glycans that are involved in the pathogenesis of IgA nephropathy should be avoided in therapeutic settings to reduce the risk of adverse side effects like the formation of anti-glycan antibodies (Novak et al. 2011).

Successful functional expression of a fully assembled recombinant secretory IgA (CaroRX™) to prevent dental caries has first been shown in transgenic *N. tabacum* (Ma et al. 1995). More recently, the production of IgA variants in different plant species displaying no apparent difference in assembly, integrity, and functionality compared to mammalian-produced IgA has been reported (Karnoup et al. 2005; Paul et al. 2014; Göritzer et al. 2017; Dicker et al. 2016). The glycosylation efficiency is essentially the same in plant and mammalian expression systems with an almost complete occupancy of N-glycans on all sites of IgA except the one in the C-terminal tailpiece (Göritzer et al. 2017). The N-glycan diversity found on plant-produced recombinant IgAs is, however, reduced compared to mammalian-derived IgA, with biantennary complex-type structures like $GlcNAc_1Man_3GlcNAc_2$ and GnGn as major glycoforms. In contrast, tri- and tetra-antennary structures, bisecting GlcNAc, and capping with sialic acid, which can be detected on mammalian-derived IgA (Royle et al. 2003; Mattu et al. 1998), are missing in plants. Furthermore, it has been reported that IgA transiently produced in *N. benthamiana* displays variable amounts of oligomannosidic structures indicating inefficient secretion (Paul et al. 2014; Westerhof et al. 2014; Göritzer et al. 2017). Site-specific N-glycan analysis revealed major differences between the individual N-glycosylation sites of each IgA subtype. These distinct features are conserved among the different IgA subtypes and expression systems, although their glycosylation repertoire is very different. The most pronounced difference is the complete lack of α1,6- and α1,3-linked core fucose in the CH2-resident N-glycosylation site in all IgA isotypes expressed in mammalian cells and wild-type plants, respectively (Göritzer et al. 2017; Dicker et al. 2016). N-glycans found on dimeric IgA produced in plants are similar but display a shift from paucimannosidic structures to more processed structures (GnGn) compared to

their monomeric counterparts (Göritzer et al. 2020). This trimming likely occurs in a post-Golgi compartment by β-hexosaminidases and further differences between monomeric and dimeric IgAs can be explained by changes in the accessibility of the N-glycans due to dimer formation and incorporation of the JC. Like the JC of mammalian-derived IgA, the N-glycosylation site is fully occupied but displays high amounts of oligomannosidic N-glycans that are not commonly detected on the JC of dimeric IgA produced in mammalian cells (Paul et al. 2014; Göritzer et al. 2020). In humans, the SC of mucosal IgA is heavily glycosylated with branched complex N-glycans carrying high levels of sialic acid and the seven putative sites are occupied in varying degrees (Huang et al. 2015). A comprehensive site-specific and quantitative N-glycan analysis of the SC incorporated in plant-produced IgA is still lacking. Partial analysis revealed differing data on the N-glycan profile with either mostly complex-type structures or the presence of oligomannosidic structures indicating different subcellular trafficking routes of distinct IgA variants (Paul et al. 2014; Westerhof et al. 2014; Dicker et al. 2016).

The most significant difference between plant and mammalian expression hosts is the modification of the proline-rich hinge region. O-glycans found on IgA1 produced in mammalian cells are a combination of mucin-type core structures with a maximal occupation of six out of nine potential O-glycosylation sites (Göritzer et al. 2017; Royle et al. 2003). On the hinge region of plant-produced recombinant IgA1 the conversion of proline residues located next to O-glycosylation sites to Hyp and the presence of additional pentoses, presumably representing attached arabinose chains has been detected in different plant-based systems (Karnoup et al. 2005; Göritzer et al. 2017). These protein modifications increase the heterogeneity of plant-produced proteins, impede a detailed site-specific analysis of engineered O-glycan analysis, and may have adverse properties that affect the functionality or immunogenicity of therapeutic IgA.

One of the most important steps toward humanizing IgA1 antibodies produced in plants is the successful modification of the hinge-region with disialylated mucin-type core 1 O-glycans that largely resemble the human serum glycoform (Dicker et al. 2016). The recently completed sequencing of the glycoengineered *N. benthamiana* ΔXT/FT line (Schiavinato et al. 2019) allows now thorough mining of P4H candidates responsible for the conversion to Hyp to set up genome editing approaches for their elimination. Other shortcomings of plant-produced IgA such as underglycosylation of the IgA tailpiece as well as the presence of paucimannosidic structures could be counteracted applying similar glycoengineering approaches as described for plant-produced IgG. It is possible to overcome the reduced glycosylation efficiency by co-expression of the single subunit OST from *L. major* (LmSTT3D) (Göritzer et al. 2020; Castilho et al., 2018). A higher occupancy of the tailpiece N-glycan also increased the efficiency of dimeric IgA assembly in plants. Furthermore, through co-expression of human GnTII, the ratio of fully processed structures with two terminal GlcNAc residues (GnGn) can be substantially increased in *N. benthamiana* ΔXT/FT. In a recent study, the N-glycan core structure of monomeric IgA produced in the *N. benthamiana* ΔXT/FT could be further extended carrying terminally galactosylated and sialylated N-glycans with high

homogeneity at each N-glycosylation sites of all IgA isotypes through co-expression of the respective mammalian glycosylation enzymes (Göritzer et al. 2019). Additionally, the generation of monomeric IgA variants carrying mainly truncated paucimannosidic glycans could be achieved through overexpression of two β-hexosaminidases targeted to the *trans*-Golgi and apoplast. The produced glycovariants were then used to investigate the influence of distinct glycoforms on conformational and thermal stability as well as binding to FcαRI, the main IgA receptor. Consistent with data from human serum IgA, no effect on FcαRI binding was observed for the plant-produced IgA glycoforms (Mattu et al. 1998; Göritzer et al. 2019). On the other hand, a recent study reported that removal of terminal sialylation from serum IgA1 increases its pro-inflammatory capacities and distinct site-specific glycan modifications could play a role for effector functions (Steffen et al. 2020). Further studies with glycoengineered plant-produced monomeric and polymeric IgAs will contribute to shed light on the function of distinct IgA glycan modifications.

16.7 Glycosylation of Plant-Produced IgEs

IgE is the least abundant serum antibody and a central player in the allergic response. IgE antibodies directed toward allergens lead to symptoms of allergy through binding to the high-affinity IgE receptor FcεRI. The IgE structure differs from IgG in that IgEs contain four constant domains compared to three constant domains in IgG classes (Arnold et al. 2007) (Fig. 16.2). IgE is the most heavily N-glycosylated antibody with seven N-glycosylation sites distributed across the constant domain of the human ε-(heavy) chain. Five sites are predominately occupied by complex N-glycans containing core fucose and different levels of sialic acids (Arnold et al. 2004; Plomp et al. 2014; Shade et al. 2015; Montero-Morales et al. 2017). Asn383 is not glycosylated on recombinant, myeloma or serum IgE and Ans394 carries exclusively oligomannosidic N-Glycans. The N-glycan at this site corresponds to Asn297 from the IgG1 heavy chain and occupies the cavity between two Fc domains (Wurzburg et al. 2000). While removal of the N-glycan at Asn394 impairs effector functions (Shade et al. 2015; Jabs et al. 2018), specific modifications of the other N-glycans did not result in altered FcεRI binding on mast cells (Montero-Morales et al. 2019).

A recombinant human IgE antibody targeting HER2 has been transiently expressed in *N. benthamiana* and compared to the same antibody produced in HEK293 cells (Montero-Morales et al. 2017). Like the human cell-derived variant, plant-produced IgE carried complex N-glycans at the same N-glycosylation sites, Asn383 was not occupied and Asn394 was modified with oligomannosidic N-glycans. When produced in the glycoengineered ΔXT/FT line, the majority of the N-glycans on these sites correspond to GnGn. N-glycosylation sites Asn140, Asn168, Asn265, and Asn394 were essentially fully glycosylated. By contrast, Asn218 and Asn371 displayed underglycosylation with 18–48% occupancy

compared to 75–90% in human cell-derived IgE (Castilho et al. 2018). Co-expression of the single subunit OST LmSTT3D increased the occupancy at both sites and resulted in more than 60% glycosylation of Asn383 with a complex-type N-glycan. Moreover, transient expression of recombinant IgE in *N. benthamiana* capable of protein sialylation resulted in N-glycans with terminal sialic acid ranging from 45 to 78% (Montero-Morales et al. 2019). The sialylation content of IgEs differs in individuals with specific allergies and allergic reactions may be attenuated by reduced levels of sialylated IgEs (Shade et al. 2020). Recombinant IgE variants with different amounts of sialic acid are therefore valuable for the characterization of distinct IgE functions.

16.8 Glycosylation of Plant-Produced IgMs

IgMs are the first antibodies produced during a humoral immune response and the third most abundant antibody subclass in humans. IgMs are heavily glycosylated oligomers containing five N-glycosylation sites on each IgM μ-(heavy) chain (Asn171, Asn332, Asn395, Asn402, and Asn563) (Arnold et al. 2007) (Fig. 16.2). In human serum, IgMs circulate mainly as pentamers consisting of 10μ-chains, 10 light chains, and a single JC that are linked by disulfide bridges. Together with the single N-glycosylation site in the JC, a pentameric IgM has 51 potential N-glycosylation sites. In addition, IgM can occur as a hexamer with 60 potential N-glycosylation sites. Like IgE, the μ-chain has four domains in the constant region. Asn171 is in the CH1 domain, Asn332 in the CH2 domain, Asn395 as well as Asn402 are located in the CH3 domain. Asn563 is located in the C-terminal tailpiece region which is required for JC incorporation and multimerization (Wiersma et al. 1998). On human serum, IgM and recombinant pentameric IgM, Asn171, Asn332, and Asn395 carry predominately biantennary complex N-glycans with different degrees of sialylation (Loos et al. 2014; Pabst et al. 2015; Moh et al. 2016; Chandler et al. 2019; Hennicke et al. 2020). By contrast, Asn402 displays mainly $Man_5GlcNAc_2$ structures and Asn563 $Man_6GlcNAc_2$ to $Man_8GlcNAc_2$ oligomannosidic N-glycans. While sites Asn171, Asn332, Asn395, and Asn402 are typically fully occupied with N-glycans, there is some variation in the glycosylation efficiency of Asn563. On human serum-derived IgM or recombinantly produced IgM, full glycosylation (Loos et al. 2014; Pabst et al. 2015) as well as reduced N-glycosylation efficiency with only 17–60% occupancy were reported for Asn563 (Arnold et al. 2005; Moh et al. 2016; Chandler et al. 2019).

Previous studies have shown that IgM N-glycans are functionally important. Abolishing N-glycosylation impacts IgM secretion (Sitia et al. 1984) and immunomodulatory effects such as the internalization of IgM by T cells (Colucci et al. 2015) or complement activation (Wright et al. 1990; Gadjeva et al. 2008). On the other hand, distinct glycan modifications appear dispensable for the binding to the human Fcμ receptor (Lloyd et al. 2017).

Transient co-expression of the μ, light, and joining chains in *N. benthamiana* resulted in the expression of a functional IgM with a high proportion of hexamers

(Loos et al. 2014). The type of N-glycans found on plant-produced IgM resembled that of recombinant IgM derived from human cells. Glycosylation sites Asn171, Asn332, Asn395 carried more than 50% of complex GnGn N-glycans when expressed in the glycoengineered ΔXT/FT line. On sites Asn402 and Asn563 96% of oligomannosidic N-glycans were detected. Upon co-expression of the pathway for *in planta* protein sialylation, complex N-glycans with mono- and disialylated structures were present on sites Asn171, Asn332, and Asn395 (Loos et al. 2014). Although the N-glycosylation efficiency at site Asn563 of plant-produced IgM was not reported, it is likely that the site in the tailpiece is incompletely glycosylated. As described for plant-produced dimeric IgA, the reduced N-glycan occupancy may affect the JC incorporation and leads to the higher proportion of hexameric IgM in plants (Loos et al. 2014).

16.9 Conclusion and Outlook

In the last couple of years, a comprehensive glycosylation analysis of all antibody subclasses (except IgD) produced in plants has been performed. Overall, the analysis revealed that the type of N-glycans (complex vs. oligomannosidic) are conserved when expressed in plants. Differences are found due to the simplified N-glycan processing pathway, the sometimes reduced N-glycosylation efficiency and the completely missing mucin-type O-glycosylation pathway. *N. benthamiana* plants are amenable to glycoengineering that resulted in the production of different recombinant antibodies with quite homogenous human-like glycans. These tailored structures are essential to investigate the biological function of distinct glycan modifications and make plants an attractive platform for the generation of recombinant antibodies with diverse activities and applications (Wang and Ravetch 2019).

Compliance with Ethical Standards

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Ethical Approval This chapter is a review of previously published accounts, as such, no animal or human studies were performed.

Conflict of Interest Kathrin Göritzer declares that she has no conflict of interest. Richard Strasser declares that he has no conflict of interest.

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Chapter 17
The Rapidly Expanding Nexus of Immunoglobulin G N-Glycomics, Suboptimal Health Status, and Precision Medicine

Alyce Russell and Wei Wang

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Abstract Immunoglobulin G is a prevalent glycoprotein, whose downstream immune responses are partially mediated by the N-glycans within the fragment crystallisable domain. Collectively termed the N-glycome, it is considered a complex intermediate phenotype: an amalgamation of genetic predisposition, environmental exposure, and health behaviours over the life-course. Thus, the immunoglobulin G N-glycome may provide an indication of health status on the spectrum from health to disease and infirmary. Although variability exists within and between populations, composition of the immunoglobulin G N-glycome remains stable over short periods of time. This underscores the potential of harnessing the immunoglobulin G N-glycome as an ideal tool for preclinical disease risk prediction, stratification, and prognosis through the development of precise dynamic biomarkers.

Keywords Immunoglobulin G N-glycome · Biomarker · Precision medicine · Suboptimal health

A. Russell · W. Wang (✉)
Centre for Precision Health, Edith Cowan University, Joondalup, Australia

School of Medical and Health Sciences, Edith Cowan University, Joondalup, Australia
e-mail: a.russell@ecu.edu.au; wei.wang@ecu.edu.au

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Abbreviations

A/G Ratio	Android/Gynoid Ratio
AD	Alzheimer's disease
ADCC	Antibody-dependent cell cytotoxicity
BMI	Body mass index
BP	Blood pressure
CCA	Canonical correlation analysis
C_H	Constant heavy
C_L	Constant light
CRP	C-reactive protein
Fab	Fragment antigen binding
FBG	Fasting blood glucose
Fc	Fragment crystallisable
FcR	Fragment crystallisable receptor
gQTL	Quantitative trait loci of N-glycosylation
GWAS	Genome-wide association study
HDL	High-density lipoprotein
IBD	Inflammatory bowel disease
ICD	International Classification of Diseases
IgG	Immunoglobulin G
LDL	Low-density lipoprotein
MBL	Mannose-binding lectin
MetS	Metabolic syndrome
PD	Parkinson's disease
PTM	Post-translational modification
RA	Rheumatoid arthritis
SHS	Suboptimal health status
SLE	Systemic lupus erythematosus
T2DM	Type-2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
UPLC	Ultra-performance liquid chromatography
V_H	Variable heavy
V_L	Variable light
WHO	World Health Organisation
WHR	Waist-to-hip ratio
WHtR	Waist-to-height ratio

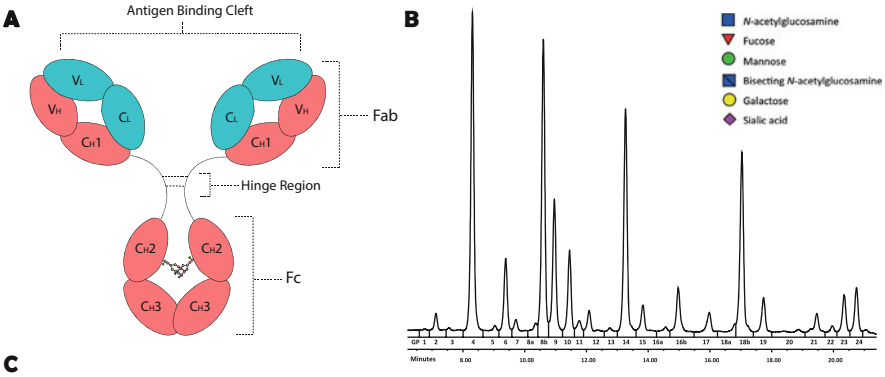
17.1 IgG N-Glycans in the Context of Biomedical Research

The premise of the Human Genome Project, a collaborative international effort launched in the 1990s, was to explore the genetic underpinnings of a plethora of human phenotypes and identify causal genes of congenital and chronic diseases (Collins and McKusick 2001). Although most phenotypes were inevitably too complex to be explained by genetics alone, the Human Genome Project set precedence for ethical considerations with open-source databases and research. Importantly, the Consortium elucidated few genes than first thought, with approximately 24,000 identified instead of the hypothesised 100,000 (Salzberg 2018). These outcomes lend support to the emerging field of epigenetics, which are heritable modifications to gene expression rather than the genome itself, and the importance of post-translational modifications (PTM), among others, in the construction of the complex phenotypes in the human repertoire.

Protein N-glycosylation, a process involving the addition of complex branching carbohydrate moieties (known as N-glycans) to proteins, is an important PTM that affects many biological processes, including those underlying human disease (Lauc et al. 2013; Russell et al. 2018). The full significance of the timing, presence, and function of the diverse range of glycoproteins is the focus of abundant cutting-edge research, not least because of the realisation that the complexity of the human glycoproteome is several orders of magnitude greater than its proteome (Lauc et al. 2013). Protein N-glycosylation is important in many vital biological processes such as cell adhesion, protein folding, molecular trafficking and clearance, receptor activation, and signal transduction (Russell et al. 2018; Li et al. 2019). More than half of the plasma proteins are N-glycosylated, including the antibody immunoglobulin G (IgG), whose N-glycans are crucial for conformation of the fragment crystallisable (Fc) region, which mediates downstream immune responses (Russell et al. 2018).

IgG is an important effector glycoprotein linking the innate and adaptive branches of the immune system. The protein portion of IgG consists of four polypeptide chains: two identical light chains, which may be kappa or lambda light chains, and two identical heavy chains (Fig. 17.1a) (Vidarsson et al. 2014). These are held together by intra-peptide disulphide bonds that cause the formation of loops and link the anti-parallel β-sheets in the tertiary structure of IgG (Vidarsson et al. 2014). The IgG glycoproteins contain two functionally distinct regions: the fragment antigen-binding (Fab) and the Fc. The Fab contains the variable (V_L and V_H) and constant (C_L and C_H1) light and heavy chain domains. These form the antigen-binding cleft, with the highly variable polypeptide sequences complementing specific target antigens (Vidarsson et al. 2014), whereas the Fc consists of constant heavy chain domains (C_H2 and C_H3) and mediates key effector functions (Pincetic et al. 2014).

The IgG Fc orchestrates various immune responses, which are either independent from antigen-binding (e.g. anti-inflammatory activity of intravenous immunoglobulins) or triggered by antigen recognition and dependent on the affinity for a number



C

GP	Abbreviated Structure	Pictured Structure	%	GP	Abbreviated Structure	Pictured Structure	%	GP	Abbreviated Structure	Pictured Structure	%
GP1	FA1			GP9	FA2[3]G1			GP17	A2G2S1		
GP2	A2			GP10	FA2[6]BG1			GP18	A2BG2S1		*
GP3	A2B			GP11	FA2[3]BG1				FA2G2S2		
GP4	FA2			GP12	A2G2			GP19	FA2BG2S1		
GP5	M5			GP13	A2BG2			GP20	FA2FG2S1		
GP6	FA2B			GP14	FA2G2			GP21	A2G2S2		
GP7	A2[3]G1			GP15	FA2BG2			GP22	A2BG2S2		
GP8	A2BG1		*	GP16	FA2[6]G1S1		*	GP23	FA2G2S2		
	FA2[6]G1				FA2[3]G1S1			GP24	FA2BG2S2		

Fig. 17.1 The immunoglobulin G (IgG) N-glycome. (**a**) IgG contains two functionally distinct regions, linked via a protein scaffold hinge: the fragment antigen-binding (Fab) and the fragment crystallisable (Fc). Within the IgG Fab exists the antigen-binding cleft, responsible for antigen recognition (Russell et al. 2018). (**b**) The IgG Fc has a branching N-glycan within each constant heavy 2 (C_H2) domain. Collectively known as the IgG N-glycome, these may be analysed using ultra-performance liquid chromatography (UPLC), which separates the N-glycans by retention time and allows the estimation of abundance (Trbojevic-Akmacic et al. 2017). (**c**) These UPLC-separated N-glycan peaks contain unique N-glycan structures. The glycoforms prevalent within each peak are depicted in terms of abbreviated and pictured structure, with * indicating the major structure (Pučić et al. 2011). F—core (if the first letter) or antennary fucose, A2—biantennary, B—bisecting N-acetylglucosamine, Gx—galactose, Sx—sialic acid

of activating and inhibitory Fc receptors (FcRs) and complement factors (Russell et al. 2018; Quast et al. 2017). These key immune responses include pathogen clearance, antibody-dependent cell cytotoxicity (ADCC), and complement-initiated inflammation, all with both beneficial and detrimental effects depending on the premise of the IgG glycoprotein's activity (Russell et al. 2018). For example, during primary bacterial infection, IgG can initiate opsonisation through complement activation and phagocytosis of the bacterial cells by macrophages, monocytes, and neutrophils, as well as neutralise endotoxins and exotoxins (Subedi and Barb 2015; Krause et al. 2002; Ioan-Facsinay et al. 2002). These well-established beneficial

effects have been harnessed for monoclonal antibody therapy in immunodeficient individuals (Schwab and Nimmerjahn 2013). On the contrary, there are examples where these effects are detrimental. In rheumatoid arthritis (RA) patients, IgG is thought to tandemly bind synovial cells and mannose-binding lectin (MBL), resulting in the initiation of the lectin complement cascade and secondary damage of surrounding tissues within the synovial joints (Fujita 2002; Quast and Lünemann 2014; Malhotra et al. 1995).

The IgG Fc N-glycans alter the glycoprotein's affinity for several FcRs and complement factors, which may be generalised as eliciting anti-inflammatory or pro-inflammatory responses (Russell et al. 2018; Ahmed et al. 2014). These immune responses are partially mediated by the conserved N-glycan within the C_H2 domain (Fig. 17.1a). Though most of the IgG Fc N-glycans are complex-type moieties (Russell et al. 2018), each IgG has two C_H2 chains (Fig. 17.1a). Thus, it assimilates two Fc N-glycans that often differ and interact with each other, leading to greater variability in downstream effector response (Nimmerjahn et al. 2007; Dekkers et al. 2017).

17.2 IgG N-Glycans as an Intermediate Phenotype of Health Status

IgG N-glycosylation is complex, mediated by glycosyltransferases and glycosylhydrolases that add and remove monosaccharides, respectively, from the maturing N-glycan structures within the endoplasmic reticulum and Golgi apparatus (Russell et al. 2018). The availability of these specialised enzymes is determined by the expression of hundreds of glycogenes within the producing plasma cell (Russell et al. 2017, 2018; Adua et al. 2017a; Vučković et al. 2015). Variations to the IgG N-glycans that appear structurally minute, however, can significantly alter its affinity to several FcRs and complement factors (summarised in Fig. 17.2). Numerous quantitative trait loci of N-glycosylation (gQTLs) have shown a clear directional effect with either increases or decreases in the relative abundance of certain IgG glycoforms (Lauc et al. 2013; Wang et al. 2011; Klarić et al. 2020). Though polymorphisms have been identified within these gQTLs, they cannot fully explain IgG N-glycome heterogeneity, even in very large population-based association studies. In fact, the post-translational timing of protein N-glycosylation indicates that the competing genetic underpinnings of the cell and the "cellular environment", not genetics alone, drive IgG N-glycome variability (Russell et al. 2018; Adua et al. 2017a; Wahl et al. 2018).

Several endogenous and exogenous factors are known to alter the cellular environment. These include cytokines and other immune mediators released by an array of leucocytes, not limited to those within the antibody-producing plasma cell (Russell et al. 2018; Wang et al. 2011; Horvat et al. 2011; Johnson et al. 2013; Rabinovich Gabriel and Croci 2012), and an array of complex phenotypes, including

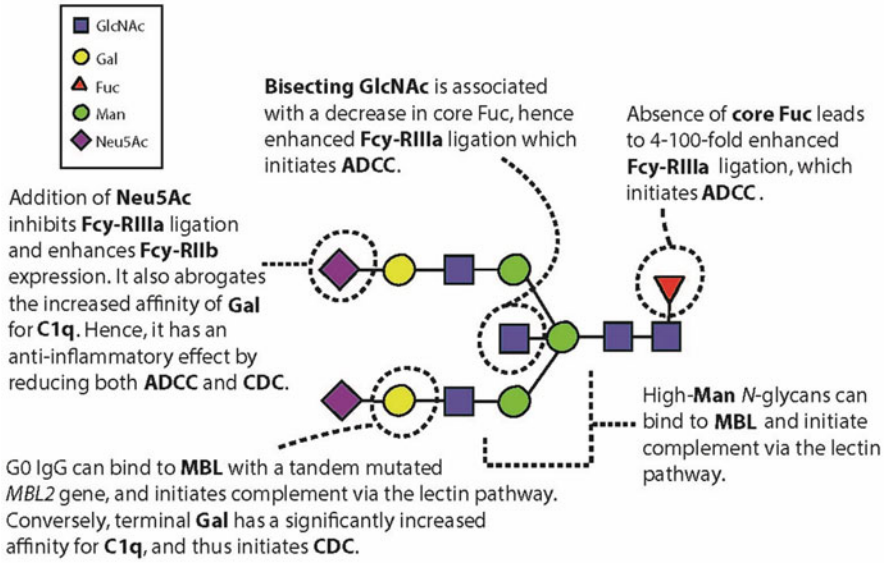


Fig. 17.2 Altered IgG N-glycosylation and its downstream effects. This figure was originally published in Russell et al. (2018) and is licensed under a Creative Commons Attribution 4.0 International License. No changes were made. GlcNAc—N-acetylglucosamine. Gal—galactose, Fuc—core fucose, Man—mannose, Neu5Ac—N-acetylneuraminic acid (sialic acid), ADCC—antibody-dependent cell cytotoxicity, CDC—complement-dependent cytotoxicity

biological and chronological age (Krištić et al. 2014; Yu et al. 2016), sex (Ercan et al. 2017), hormones levels (Ercan et al. 2017; Engdahl et al. 2017; Chen et al. 2012), and disease presence (Russell et al. 2017, 2018; Adua et al. 2017a; Vučković et al. 2015). Given its potential to identify and monitor health status, the IgG N-glycome has been implemented in several population-based studies exploring the biological mechanisms of altered disease states. A shift towards certain IgG N-glycan profiles has been reported for a number of diseases and conditions, including RA (Troelsen et al. 2012; Sebastian et al. 2016), metabolic syndrome (MetS), and type 2 diabetes mellitus (T2DM) (Lauc et al. 2013; Li et al. 2019; Lemmers et al. 2017; Adua et al. 2018; Liu et al. 2018a, 2019), inflammatory bowel disease (IBD) (Lauc et al. 2013), systemic lupus erythematosus (SLE) (Vučković et al. 2015), hypertension (Wang et al. 2016; Liu et al. 2018b), various cancers (Lauc et al. 2013; Meany and Chan 2011), and neurological disorders such as Alzheimer's disease (AD) and progressive mild cognitive impairment (Lundström et al. 2013), multiple sclerosis (Wuhrer et al. 2015), and Parkinson's disease (PD) (Russell et al. 2017). These studies report disease-specific shifts, underscoring the plausible biological importance of the IgG N-glycome in disease pathogenesis.

We consider the IgG N-glycome a complex "intermediate phenotype"; an amalgamation of genetic predisposition, environmental exposure, and health behaviours over the life-course, which provides an indication of health status on the spectrum from health to disease (Russell et al. 2018, 2019a). Although variability exists, IgG

N-glycome composition is considered a predesigned outcome of the producing plasma cell (Russell et al. 2018; Vidarsson et al. 2014) and is stable over short periods, with current estimates over 6–12 months (Pučić et al. 2011; Adua et al. 2018). This makes it an ideal target for measuring health status in the context of biomarker research and the shift towards precision medicine, which aims to diagnose early, delay further development of disease, monitor treatment efficacy, and improve quality of life through prevention and tailored interventions (Wang et al. 2014).

17.3 Defining the Grey Area Between Health and Chronic Disease

Health is the optimum level of functional or metabolic efficiency, in the absence of illness, stress, injury, or pain (Wang and Yan 2012). The World Health Organisation (WHO) defines health as "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmary" (Sartorius 2006). Though it has persisted as the most widely accepted definition of health since its dissemination in 1946, controversy has arisen due to the definition lacking operational value and the limitation created by the word "complete" (Sartorius 2006; Callahan 1973; Jadad and O'Grady 2008). A broader approach may be achieved by instead focusing on health status, which is the indication of a person's combined state of physical, mental, and social well-being (Wang and Yan 2012). Indeed, other classification systems, such as the International Classification of Diseases (ICD), place greater emphasis on identifying and measuring components of health status, which may be in different states of well-being (Wang and Yan 2012).

There has been considerable effort into defining the grey area between complete health and disease. For example, Suboptimal Health Status (SHS) has been defined as a physical state whereby no specific clinically diagnosable conditions are present (Yan et al. 2012), and focuses on identifying a decline in vitality, physiological function, and capacity for adaptation (Yan et al. 2009). SHS recognises a person's perception of health complaints, general weakness, chronic fatigue, and low energy, which may elucidate suboptimal or less-than-ideal components of health status requiring intervention (Wang et al. 2014). Early intervention is consistently regarded as the most effective method of preventing chronic disease and can be achieved through prescribing modified health behaviours or treating mild complaints from the perspectives of precision medicine.

Our initial efforts were directed at developing the Suboptimal Health Status Questionnaire-25 (SHSQ-25), which we have since validated in multiple populations, including Africans, Asians, and Caucasians (Wang et al. 2014; Wang and Yan 2012; Yan et al. 2012; Adua et al. 2017b, 2020; Anto et al. 2019; Kupaev et al. 2016). The SHSQ-25 incorporates Traditional Chinese Medicine principles, and assesses health status from the perspective of five domains: (1) the cardiovascular system, (2) the immune system, (3) the digestive system, (4) fatigue, and

(5) mental health. Subsequent research has highlighted the relationship between total and domain-specific SHS scores and many objective health indicators, including cardiometabolic risk factors such as triglycerides (TG), C-reactive protein (CRP), insulin, and alkaline phosphatase, as well as endothelial dysfunction, blood pressure (BP), obesity, plasma cortisol and glucocorticoid receptor α/β (Yan et al. 2015).

More recently, there have been investigations into the association of the total and domain-specific SHS scores and the IgG Fc N-glycans, and how these relate to complex phenotypes (Sebastian et al. 2016; Yan et al. 2012; Anto et al. 2019). Preliminary results suggest an interplay between the complexity of the IgG N-glycome, metabolic risk factors, and SHS (Liu et al. 2018c, 2019; Adua et al. 2017b). These profiles may hold the key to understanding the underlying biological mechanisms of SHS and are under further investigation (Yan et al. 2009; Lu et al. 2011). It is envisaged these findings will pave the way for preclinical disease risk prediction, stratification, and prognosis through the development of precise dynamic biomarkers, as well as identify potentially modifiable health behaviours or pharmaceutical targets for early intervention.

17.4 Unravelling IgG Fc N-Glycan Inter- and Intra-population Variability

The IgG N-glycome presents itself as a promising biomarker. Although variability exists within and between populations (Pučić et al. 2011; Štambuk et al. 2020), including between Chinese minority groups (Liu et al. 2018b), its composition is considered a predesigned and relatively stable outcome of the producing plasma cell (Russell et al. 2018; Vidarsson et al. 2014). Therefore, a plethora of research is underway to determine the IgG N-glycome's efficacy in elucidating overall health status, identifying the risk of developing disorders, tracking progression of phenotypic disorders, and indicating who may respond to certain therapies or whether a currently prescribed therapy is effective.

Our ongoing research demonstrates the discriminatory utility of the IgG N-glycome in various population studies of chronic disease and its related phenotypes, including RA (Troelsen et al. 2012; Sebastian et al. 2016), MetS and T2DM (Lauc et al. 2013; Li et al. 2019; Lemmers et al. 2017; Adua et al. 2018; Liu et al. 2018a, 2019), SLE (Vučković et al. 2015), hypertension (Wang et al. 2016; Liu et al. 2018b), central adiposity (Russell et al. 2019b), ischaemic stroke (Liu et al. 2018d), and PD (Russell et al. 2017). Although promising results are evident, considerable IgG N-glycome heterogeneity exists. Thus, they also underscore the need to identify what is driving altered IgG N-glycosylation, even among individuals with the same phenotypes. To bridge the gap in knowledge, we have explored genetic and other factors that may alter the cellular environment and, therefore, IgG Fc effector responses. Notably, these factors may be incorporated into IgG N-glycome models to improve their precision.

17.4.1 Genes

Several loci, termed gQTLs, are associated with aberrant N-glycosylation and have clear directional effects in the relative abundance of certain N-glycosylation features. The gQTL *HNF1α* was the first found to be associated with aberrant plasma glycosylation, a gene whose product is hepatocyte nuclear factor 1α (HNF1a). HNF1a is a master regulator of the expression of *FUT6* and *FUT8*. These gQTLs encode fucosyltransferases that influence multiple stages in fucosylation (Wang et al. 2011). Particularly for IgG, *FUT8* associates with core fucosylation in European Caucasian populations (Lauc et al. 2013; Wang et al. 2011). IgG glycoforms lacking core fucose have a 4- to 100-fold enhanced affinity for Fc*γ*-RIIIa, which initiates ADCC (Russell et al. 2018; Vidarsson et al. 2014).

Many other gQTLs have been identified, including the *ST6GAL1*, *B4GALT1*, and *MGAT3* genes that encode glycosyltransferases instrumental in adding sialic acid (Neu5Ac), galactose (Gal), and bisecting N-acetylglucosamine (GlcNAc) monosaccharides, respectively, to the branching N-glycans (Lauc et al. 2013; Wang et al. 2011; Bondt et al. 2014). Whereas genes previously associated with other diseases, such as autoimmune diseases and haematological cancers, have later been identified to have pleiotropy with IgG N-glycosylation and include *IL6ST-ANKRD55*, *IKZF1*, *ABCF2-SMARCD3*, *SUV420H1*, *SMARCB1-DERL3,* and *SYNGR1-TAB1-MGAT3-CACNA1I* (Lauc et al. 2013; Menni et al. 2013). Moreover, five novel gQTLs were recently identified and validated within European Caucasians: *IGH*, *ELL2*, *HLA-B-C*, *AZI1*, and *FUT6-FUT3* (Shen et al. 2017a).

We recently confirmed two gQTLs using an Australian Caucasian cohort; *ST6GAL1* and *MGAT3* (Russell 2020). These gQTLs were previously identified in Caucasians from four European populations, two islands in Croatia (Vis and Korcula), the Orkney Islands in the United Kingdom, and Sweden (Lauc et al. 2013). *ST6GAL1* encodes sialyltransferase-6 that catalyses the transfer of Neu5Ac to the branching N-glycan moieties (Kuhn et al. 2013). This glycosyltransferase is localised in the membrane of the Golgi apparatus and is specific to B cells in the later stages, following differentiation into antibody-producing plasma cells (Kuhn et al. 2013). Mutations within *ST6GAL1* are consistently acknowledged in genome-wide association studies (GWAS) of IgG N-glycosylation (Lauc et al. 2013; Wahl et al. 2018; Shen et al. 2017a). Indeed, this gQTL associates with the relative abundance of various sialylated IgG glycoforms. Polymorphisms within *ST6GAL1* have pleiotropy to T2DM (Lauc et al. 2013). Interestingly, altered sialylation of IgG has been implicated in T2DM among European Caucasians (Lemmers et al. 2017), Han Chinese (Wu et al. 2020), and Uyghur Chinese (Liu et al. 2019). Thus, this finding may have biological significance.

The gQTL containing *SYNGR1-TAB1-MGAT3* is another commonly acknowledged gQTL (Lauc et al. 2013; Wahl et al. 2018; Shen et al. 2017a). The strongest associate genetic polymorphism we identified was within the *MGAT3* genomic region; a gene encoding mannosyl (β-1,4)-glycoprotein β-1,4-*N*-acetylglucosaminyltransferase which adds bisecting GlcNAc to the branching IgG

glycoforms. This polymorphism associated with increased relative abundance of bisect-type IgG N-glycans among fucosylated disialylated N-glycans (Russell 2020), which has been implicated in E-cadherin, EGF-, Wnt- and integrin- cancer-associated signalling pathways (Kohler et al. 2016). Altered MGAT3 has pleiotropy with Alzheimer's disease (Fiala et al. 2011) and inflammatory bowel disease (Klasic et al. 2018). *SYNGR1* and *TAB1* are shown to be in linkage disequilibrium with *MGAT3* and encode synaptogyrin-1 and TGF-beta-activated kinase-1, respectively. Genetic polymorphisms within *SYNGR1* have pleiotropy with schizophrenia and bipolar disorder (Verma et al. 2005; Iatropoulos et al. 2009), whereas TAB1 may have pleiotropy with colorectal cancer (Gong et al. 2018).

Though associated with various N-glycosylation features, the vast repertoire of gQTLs cannot fully explain IgG N-glycome heterogeneity, even in very large association studies. The post-translational timing of N-glycosylation supports the competing effects of genetic predisposition of an antibody-producing plasma cell and its cellular environment. Certainly, this gene–environment interaction drives the variability among IgG N-glycome profiles within and between populations (Russell et al. 2018; Adua et al. 2017a; Wahl et al. 2018).

17.4.2 Gene Expression

The IgG N-glycome is both genetically and epigenetically regulated (Russell et al. 2018; Adua et al. 2017a; Wahl et al. 2018), and cytokines and other immune factors released by an array of leucocytes, not only those factors within the antibody-producing plasma cell, associate with IgG N-glycome heterogeneity (Wang et al. 2011; Horvat et al. 2011; Johnson et al. 2013; Rabinovich Gabriel and Croci 2012). Therefore, we recently analysed whole blood RNA-Seq to explore the genetic influence on the heterogeneity of the IgG N-glycome, in terms of the transcribed genes and not just genetic polymorphisms (Russell 2020). The decision to explore whole blood mRNA rather than targeting B-cell lymphocyte and plasma cell mRNA transpired since both endogenous and exogenous factors are known to alter IgG N-glycome composition and in turn, impact the inflammatory properties of IgG.

An integrative model explored how IgG N-glycome heterogeneity could be explained by the differential expression of whole blood cells via contemporaneously analysing both datasets (14,544 expressed genes and 24 IgG N-glycan peaks; see Fig. 17.1b, c). Our optimised model explicated that 58.1% of IgG N-glycome variability was explained by variable whole blood gene expression, while only 11.4% of the mRNA variability was explained by the IgG N-glycome. The biological validity of these findings was confirmed through interrogating gene ontology (GO) enrichment terms, which represent biosynthesis and degradation-related intracellular activity as well as several IgG Fc downstream effector responses (Russell 2020).

The IgG Fc binds distinct FcR and complement factors, and these are cell, spatial, and immune response specific (Russell et al. 2018; Pincetic et al. 2014).

Additionally, FcR specificity may be influenced by several factors, including the IgG Fc N-glycans (Russell et al. 2018; Quast et al. 2017). Since whole blood gene expression was considered, our results provide further evidence that cytokines and other factors extrinsic to the differentiating B-cell and antibody-producing plasma cell may mediate IgG N-glycosylation. On the contrary, the IgG N-glycans associate with very specific downstream immune responses due to IgG Fc specificity (Wang et al. 2011; Horvat et al. 2011; Johnson et al. 2013; Rabinovich Gabriel and Croci 2012). Hence, there exists a complex interplay in the epigenetic regulation of IgG N-glycosylation.

17.4.3 Clinical Indicators

Clinical indicators are arguably the most studied determinants of IgG N-glycome variability, with many studies arising from our research team (Yu et al. 2016; Sebastian et al. 2016; Wang et al. 2016; Liu et al. 2018d) and others (Krištić et al. 2014; Ercan et al. 2017; Baković et al. 2013; Keser et al. 2017). Although genetics plays a key role in the baseline risk of the chronic and altered relative abundances of IgG N-glycan features, the N-glycosylation of IgG Fc is regulated by several endogenous and exogenous factors, including routinely measured blood and clinical indicators. In this sense, the IgG N-glycome is malleable as it is reliant on the expression levels of the glycosyltransferases and glycosylhydrolases, as well as the abundance of sugar nucleotide donors, during biosynthesis.

An increase in pro-inflammatory IgG glycoforms associates with unfavourable levels of several cardiometabolic indicators, such as TG, total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Plomp et al. 2017), body mass index (BMI) and central adiposity (Russell et al. 2019b; Perkovic et al. 2014), fasting blood glucose (FBG) (Lemmers et al. 2017; Ge et al. 2018), BP (Wang et al. 2016), and CRP (Plomp et al. 2017). Very recently, IgG N-glycome heterogeneity was linked with the 10-year atherosclerotic cardiovascular disease risk score, which is a sex- and race-specific risk assessment derived using a combination of these clinical indicators (Menni et al. 2018).

We further underscored the association between pro-inflammatory IgG N-glycans and poorer health status using canonical correlation analysis (CCA); a multivariate form of correlation analysis that simultaneously associates all the IgG N-glycans with the multiple clinical indicators (Russell et al. 2019a). CRP had the strongest independent association with increased relative abundance of pro-inflammatory IgG glycoforms (Fig. 17.3). Importantly, CRP is a non-specific marker of systemic inflammation whose activity ranges from acute-phase reactions through low-grade chronic inflammation (Segman and Stein 2015), whereas the IgG N-glycome has unique signatures for various phenotypes. Both CRP and the IgG N-glycome are biomarkers of the cardiometabolic cluster of disorders, including hypertension, cardiovascular disease, ischaemic stroke, MetS, and T2DM (Wang et al. 2016; Liu et al. 2018b, d; Menni et al. 2018; Segman and Stein 2015). However, it is prudent to

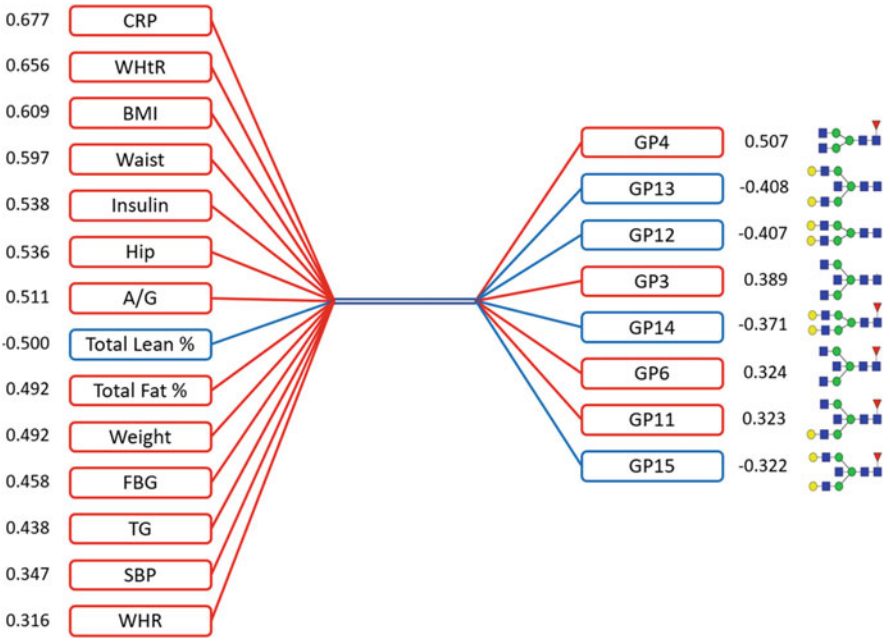


Fig. 17.3 Canonical structures of IgG N-glycan peaks (GPs) and clinical factors in the first canonical set. An absolute value of canonical loadings greater than 0.3 was considered significant. All variables are sorted by the absolute value of the canonical loadings. Positive relationships are in red, while negative relationships are in blue. No changes were made. *A/G* android-to-gynoid ratio (measured using DEXA), *BMI* body mass index, *CRP* C-reactive protein, *DEXA* dual-energy X-ray absorptiometry, *FBG* fasting blood glucose, *Hip* hip circumference, *SBP* systolic blood pressure, *TG* triglycerides, *Total Fat %* percentage of fat mass (total body), *Total Lean %* percentage of lean mass (total body), *Waist* waist circumference, *WHR* waist-to-hip ratio, *WHtR* waist-to-height ratio. Reprinted with permission from Russell et al. (2019a)

underscore the novelty of the IgG N-glycome, which has unique signatures for many phenotypes when compared with CRP, known for its non-specific indication of the presence of inflammation. Thus, although both pro-inflammatory IgG N-glycans and CRP are present in low-grade chronic inflammation, the IgG N-glycome elucidates more specific information about the underlying biology of preclinical and clinical diseases (Russell et al. 2017; Lemmers et al. 2017; Wang et al. 2016; Menni et al. 2018; Bondt et al. 2013; Dekkers et al. 2018).

CRP positively associates with adipokines released from central body fat, particularly visceral fat; an endocrine organ in its own right (Fontana et al. 2007). Increased central adiposity compared with hip fat (as measured with dual energy X-ray absorptiometry-derived android-to-gynoid ratio; A/G ratio) or height (waist-to-height ratio; WHtR) are strongly associated with pro-inflammatory IgG glycoforms, more so than BMI (Russell et al. 2019b). Moreover, visceral body fat produces interleukin-6 (IL-6), to a greater degree than subcutaneous fat (Panagiotakos et al. 2005), and associates with increased levels of an array of

systemic inflammatory markers (Hsieh et al. 2014; Gaens et al. 2015), including CRP (Fontana et al. 2007). Chronic, subclinical inflammation has been linked to obesity (Festa et al. 2001; Alissa et al. 2016), and central adiposity is more detrimental than total body fat percentage; obese individuals (according to BMI guidelines) with low A/G ratios are less likely to develop MetS (Koster et al. 2010).

Central adiposity is a major risk factor of disorders such as MetS (Koster et al. 2010), cardiovascular disease (Shen et al. 2017b), and AD (Whitmer et al. 2008), while the IgG N-glycome has utility in identifying disease presence and severity (Lemmers et al. 2017; Wang et al. 2016; Liu et al. 2018d; Koster et al. 2010; Shen et al. 2017b; Novokmet et al. 2014). CCA further validated previously identified univariate associations, evidencing four measures of central adiposity to be moderately to strongly associated with the IgG N-glycome, namely WHtR, BMI, waist circumference, and waist-to-hip ratio (WHR) (Fig. 17.3). Indeed, IgG N-glycosylation heterogeneity may be an immunological response to centrally located body fat (Russell et al. 2019b). However, it remains to be determined whether increases in central adiposity cause the increase in pro-inflammatory IgG glycoforms, or whether it is reverse causation or bidirectional (Russell et al. 2019b).

17.5 The IgG N-Glycome in the Precision Medicine Framework

The recently initiated Human Glycome Project aims to follow in the footsteps of the Human Genome Project and elucidate biological regulation within the human body, leveraged by the collaborative effort of world leaders in glycobiology from several countries (Bennett 2019; Wang 2019). Moving beyond laboratory- and population-based studies, there has been a surge in effort focused on translating glycobiology research into the clinical practice (Fig. 17.4). Currently, clinical tests are emerging in Europe and China that quantify biological age using the dynamic IgG N-glycome, following results from several studies (Krištić et al. 2014; Yu et al. 2016; Vanhooren et al. 2010; Ruhaak et al. 2010). The premise of "GlycanAge" is to predict biological age, thought to combine genetic predisposition with the effects of several health behaviours and environmental exposures over the life-course. Therefore, an individual may use this to identify potential discordance from their chronological age. It is an important initiative in the context of precision medicine. However, it remains to be determined how individuals will truly benefit from the knowledge of subclinical morbidity risk or discordant biological age, particularly if much higher than chronological age.

Variation among several clinical indicators, particularly those related to cardiometabolic health, associate with the heterogeneity of IgG N-glycosylation, and may represent instrumental variables of health behaviours and environmental exposure (Lauc et al. 2013; Li et al. 2019; Liu et al. 2018a, d; Wang et al. 2016; Russell et al. 2019a, b; Menni et al. 2018). By virtue of this gene–environment

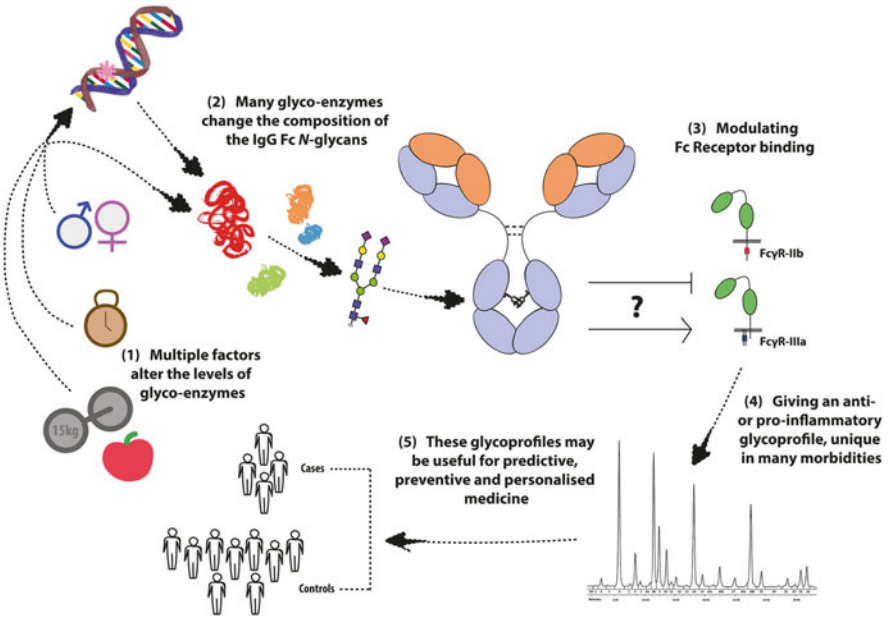


Fig. 17.4 Use of the IgG N-glycome in the context of precision medicine. This figure was originally published in Russell et al. (2018) and is licensed under a Creative Commons Attribution 4.0 International License. No changes were made

interplay, it may be possible to remodel IgG N-glycan composition by modifying certain negating health behaviours associated with poorer health outcomes (Russell 2020). Lowering or avoiding certain health behaviours is heavily promoted as a vector to improve overall health status and increase disease-free life. Aside from obesity and hypertension, other prominent modifiable health behaviours include physical inactivity, daily smoking, excessive alcohol consumption, inadequate consumption of fruits and vegetables, and consumption of whole milk (AIHW 2012). In fact, these seven health behaviours contribute to three-quarters of the total burden of chronic disease in Australia (AIHW 2012), as well as decline in cardiometabolic health, especially in middle-aged and elderly individuals (Díaz-Redondo et al. 2015). If shown to be causative, an adjustment in health behaviours may be prescribed to alter IgG N-glycome composition and improve health outcomes.

We have done preliminary analyses on three of these health behaviours: excessive alcohol consumption, current smoking status, and physical inactivity. There was no evidence of an effect between IgG N-glycosylation and the level of self-reported physical activity (Russell 2020). However, binge drinking and excessive alcohol consumption were associated with an increased relative abundance of pro-inflammatory IgG glycoforms (Russell 2020). Also, several IgG N-glycans were associated with current smoking status compared with those who have never smoked (Russell 2020). Smoking was previously implicated in a European-based

study (Wahl et al. 2018). Wahl et al. (2018) identified the potential mechanism whereby the association between smoking and IgG N-glycosylation may be partially mediated by altered DNA methylation at several gQTLs (Wahl et al. 2018). Importantly, we found no difference in IgG N-glycan profiles when comparing those that have never smoked to ex-smokers (Wahl et al. 2018). This may support the positive effects on health status after quitting smoking, reflected in other health research (Knuchel-Takano et al. 2018). Though changes to these modifiable health behaviours make an ideal intervention strategy, it should be noted an interplay between these health behaviours, mental status (particularly untreated mental disorders), and addiction complicates the prospect of intervening (Verdurmen et al. 2005; Teesson et al. 2010).

In conclusion, we consider the IgG N-glycome a complex intermediate phenotype, which may be harnessed as a biomarker for measuring underlying biological processes on the spectrum from health to disease and infirmary. IgG N-glycome composition varies within and between populations, is stable over short periods, and associates with various subclinical and clinical phenotypes. We further suggest it may be possible to remodel IgG N-glycan composition through modifying certain negating health behaviours. Whether an adjustment in modifiable health behaviours may improve overall health status and have a downstream impact on the IgG N-glycome is yet to be validated. However, the composition of the IgG N-glycome may be pertinent to the pathophysiology of existent subclinical or clinical phenotypes, making it an ideal target for measuring health status in the context of precision medicine.

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Disclosure of Interests The two authors declare they have no conflict of interest.

Ethical Approval The studies discussed in this book chapter have ethical approval from each institution. All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration and its later amendments.

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Chapter 18
Glycosylation of Antigen-Specific Antibodies: Perspectives on Immunoglobulin G Glycosylation in Vaccination and Immunotherapy

Pranay Bharadwaj and Margaret E. Ackerman

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Abstract Exciting developments have been made in understanding antibody-mediated immunity, deepening understanding of antibody effector functions increasingly recognized as critical mechanisms of action beyond antigen recognition, and significantly broadening the evidence base for the importance of these effector mechanisms across diverse infectious and autoimmune diseases. Because these activities critically depend on the specific glycoforms present on a conserved site of the IgG Fc domain, relationships between the Fc glycosylation profiles of antigen-specific antibody pools and outcomes in infectious and autoimmune disease have

P. Bharadwaj
Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH, USA

M. E. Ackerman (✉)
Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH, USA

Thayer School of Engineering, Dartmouth College, Hanover, NH, USA
e-mail: Margaret.E.Ackerman@Dartmouth.edu

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begun to be defined, pointing to the key role of this posttranslational modification as a biomarker and mechanistic modifier of antibody-mediated immunity. Here we summarize studies evaluating the profiles and activities of antigen-specific antibodies elicited by infection and vaccination as well as within the context of allo- and autoimmunity, and consider current approaches to rational modification of Fc glycans in vivo.

Keywords Immunoglobulin · Antibody · Fc domain · Glycosylation · Vaccine · Allergy · Autoimmunity · IgG · Effector function

Abbreviations

ACPA	Anti-citrullinated protein antibodies
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADE	Antibody-dependent enhancement
AMI	Antibody-mediated immunity
ASA	Antigen-specific antibody
CDC	Complement-dependent cytotoxicity
COVID-19	Coronavirus disease 2019
CSR	Class switch recombination
DC	Dendritic cell
DENV	Dengue virus
EndoS	Endoglycosidase S
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fc receptor
FNAIT	Fetal or neonatal allo-immune thrombocytopenia
Fv	Fragment variable
GlcNAc	N-acetylglucosamine
HPA-1a	Human platelet antigen 1a
IdeS	IgG digesting enzyme S
IgG	Immunoglobulin G
IVIg	Intravenous immunoglobulin
K	Kell
mAb	Monoclonal antibody
MAC	Membrane attack complex
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
NK	Natural killer
HIV	Human immunodeficiency virus
RA	Rheumatoid arthritis
RhD	Rhesus D

RSV	Respiratory syncytial virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

18.1 Introduction

Evidence of the critical importance of effector functions to antibody-mediated immunity (AMI) has been accumulating across studies of diverse infectious and autoimmune diseases. The ability to study and clinically leverage AMI was made a much simpler task after Kohler and Milstein's (Köhler and Milstein 1975) discovery of an approach to generate consistent, reliable, and reproducible monoclonal antibody (mAb) preparations, which represented challenges to the use of polyclonal sera prevalent at that time. Advanced molecular methods in antibody cloning (Wang et al. 2019; Hunter et al. 2019; Kim et al. 2014; Winzeler and Wang 2013; Chon and Zarbis-Papastoitsis 2011) and engineering (Bruggeman et al. 2018; Dekkers et al. 2018; Crooks et al. 2018; Dekkers et al. 2016) have complemented the discovery of diverse Fc receptors (FcR) (Bournazos and Ravetch 2017; Castro-Dopico and Clatworthy 2016; Wu et al. 2014; Hirvinen et al. 2013; Nimmerjahn and Ravetch 2008a; Lazar et al. 2006; Hogarth 2002) and development of elegant knockout mouse models (Walsh et al. 2016; Verkoczy 2017; Stackowicz et al. 2020) to enable further basic science exploration and to support therapeutic optimization of AMI. In parallel, higher resolution means of profiling serum antibodies have accompanied these advances and greatly expanded the ability to interrogate polyclonal responses in serum and tissue. The high throughput of many of these profiling approaches has now turned the heterogeneity observed among polyclonal samples into a strength, providing means to interrogate the features and activities of antibodies that are associated with AMI.

18.2 Antibody Effector Functions

Antibodies play an important role in both effecting and regulating an immune response. They have the capacity to either amplify or dampen an inflammatory immune response based on their specificity, affinity, titer, isotype, and glycosylation profile. While the antigen-binding fragment (Fab) domain confers antigen specificity, the crystallizable fragment (Fc) domain is responsible for linking antigen recognition to downstream effector functions (Schroeder and Cavacini 2010). Antibodies can neutralize pathogens by directly binding through the Fab domain and occluding the binding of the pathogen or its toxins to cognate receptors. Such Fab-mediated antibody action is complemented by Fc domain engagement of complement proteins and FcR (Fig. 18.1). There are broadly two categories of FcRs—activating and inhibitory—that are ubiquitously expressed on human hematopoietic

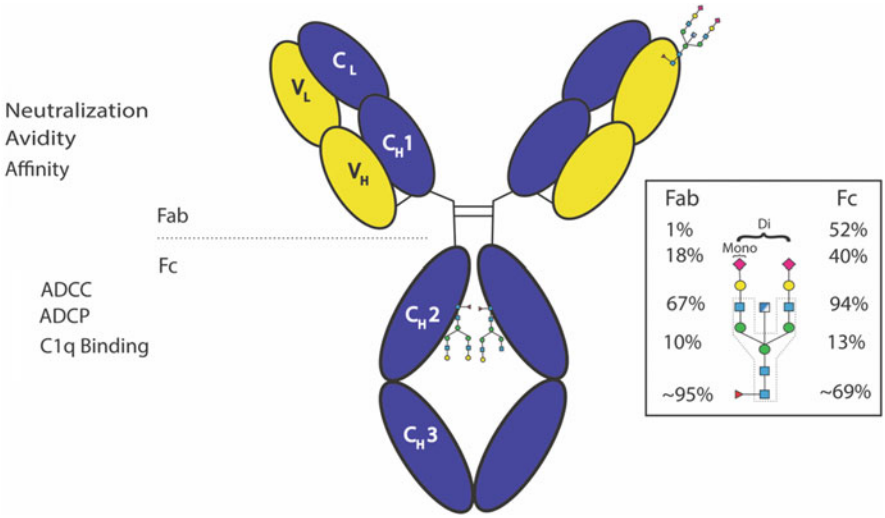

Fig. 18.1 Antibody glycosylation sites in IgG. Glycosylation sites can be present in the Fab region (15–25% of IgGs), but are always present in the Fc region. There are distinct qualitative differences among the glycans commonly found at these sites, however, the importance of the glycans in mediating function via respective antibody domains is a shared characteristic. These glycans share the core heptasaccharide (dotted line in the inset) to which extensions of specific sugars are attached. Fc glycans tend to be heavily fucosylated whereas the Fab glycans have generally been observed to exhibit relatively high levels of sialylation. Adapted from Bondt et al. (2014)

cells and play a key role in orchestrating potent antibody-mediated effector immune responses in the context of both protective immunity to pathogens and pathogenic immune responses to self (Fig. 18.2).

The most widely studied Fc-mediated antibody effector functions are antibody-dependent cell-mediated cytotoxicity (ADCC) (Worley et al. 2018), antibody-dependent cellular phagocytosis (ADCP) (Gerber and Mosser 2001), and complement-dependent cytotoxicity (CDC) (Goldberg and Ackerman 2020). These activities are induced by engagement of the FcγRs on innate effector cells, or by soluble complement cascade initiators, such as C1q or Mannose Binding Lectin (MBL), by the Fc domain of antibodies that are bound to a target antigen. ADCC is characterized by FcγR engagement that causes the release of cytotoxic granules that contain perforin and granzyme, resulting in the killing of target cells (Smyth et al. 2005). FcγRIIIA-expressing Natural Killer (NK) cells are widely considered to be an important contributor to ADCC and are often assayed in vitro. However, in vivo, neutrophils, monocytes, and macrophages are also capable of driving ADCC and have been found to make important contributions to antibody mechanism of action (Smyth et al. 2005; van Erp et al. 2019).

ADCP or opsonophagocytosis is the uptake of immune complexes or antibody-coated antigens by phagocytic cells including monocytes, macrophages, dendritic cells (DCs), and others that express FcγRI, FcγRII, and/or FcαRI, each of which can

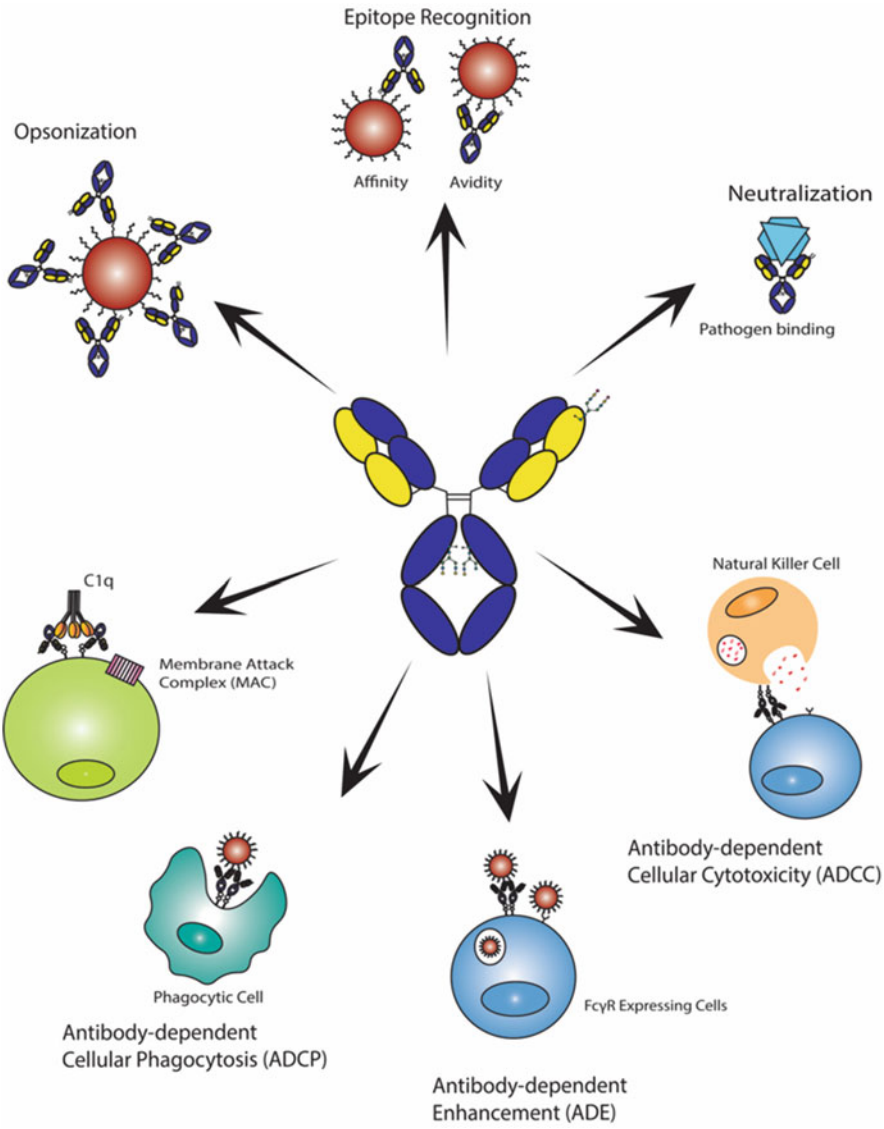

Fig. 18.2 Antibody mechanisms of action. While the Fab domain functions are directly driven by antigen recognition, Fc-mediated functions result from recruitment of various components of the innate immune system

mediate immune complex uptake (Li and Kimberly 2014). ADCP mediates clearance of immune complexes by trafficking them to the lysosomes for degradation and antigen processing for presentation on Major Histocompatibility Complex (MHC) molecules on the cell surface (Mantegazza et al. 2013). Previous work on influenza virus has shown that ADCP contributes to protection from infection in mice (Huber

et al. 2001; He et al. 2017) and potentially plays a role in recovery from severe infections in humans (Vanderven et al. 2017; Ana-Sosa-Batiz et al. 2016). Associations between ADCP and improved outcomes in other disease settings such as HIV (Barouch et al. 2013, 2015), West Nile Virus (Vogt et al. 2011) in humans, and Respiratory Syncytial Virus (RSV) (Bukreyev et al. 2012) have also been recently established.

Besides ADCC and ADCP, antibodies can also induce complement activation. The complement cascade contributes to pathogen elimination either directly, by means of complement-dependent cytotoxicity (CDC), or indirectly, through phagocytic clearance of complement-coated targets and the induction of an inflammatory response (Goldberg and Ackerman 2020; van Erp et al. 2019; Grafals and Thurman 2019; Casadevall and Pirofski 2012). The complement cascade consists of a large number of distinct plasma proteins that react with one another to opsonize pathogens, inducing a series of inflammatory responses that help to fight infection (Noris and Remuzzi 2013). A number of complement proteins are proteases that are themselves activated by proteolytic cleavage (Dunkelberger and Song 2010). The terminal complement components assemble into the membrane attack complex (MAC), resulting in lysis of the pathogen-infected cell. Complement has been shown to have both protective and pathogenic effects in various disease conditions. In HIV (Barouch et al. 2013, 2015; Pittala et al. 2019), influenza (Co et al. 2014; Wu et al. 2015), and vaccinia (Benhnia et al. 2009) infection, antibody-mediated CDC has been shown to correlate or mechanistically contribute to antibody antiviral activity. Alternatively, complement-mediated activation has also been associated with disease severity (Nascimento et al. 2009; Churdboonchart et al. 1983; Füst et al. 1994). Lastly, the binding of complement-coated immune complexes to complement receptor 2 on B cells is reported to lower the B cell activation threshold, thereby promoting long-lived adaptive immunity and higher antibody levels (van Erp et al. 2019; Hebell et al. 1991; Gonzalez et al. 2010).

18.3 Immunomodulatory Antibody Activities

In contrast, anti-inflammatory effects of antibodies can help in alleviating severe immune damage. Based on this concept, administering intravenous immunoglobulin (IVIg) to treat inflammatory conditions such as autoimmune disease has found an important clinical application (Bayry 2016). While the underpinnings of IVIg mechanisms have yet to be clearly elucidated (Schwab and Nimmerjahn 2013), different mechanisms of action such as neonatal Fc receptor blockade resulting in accelerated clearance of autoantibodies (Li and Kimberly 2014), direct interaction with the inhibitory FcγRIIb (Nagelkerke and Kuijpers 2015), or occlusion of activating receptors and tempering the inflammatory effector responses (Nimmerjahn and Ravetch 2008b) have been proposed. However, since IVIg treatment is used to treat various diseases, it is likely that the mode of action differs per clinical setting.

18.4 Induction and Regulation of Antigen-Specific Antibodies

During an immune response, B cells are stimulated to mature and to undergo class switch recombination (CSR) resulting in genetic modification of the IgH locus and selection of the antibody isotype and subclass to be secreted (Stavnezer and Schrader 2014). Just as B cells undergo rounds of somatic hypermutation over the course of affinity maturation as they migrate in and out of regions in the germinal center, CSR can occur in rounds with repeated switching to downstream types (Mesin et al. 2016). This heterogeneity in the amino acid sequence of both variable fragment (Fv) and Fc regions is coupled to further functional diversification via incorporation of one of the >30 possible glycoforms (Jennewein and Alter 2017) in the conserved N-linked glycosylation motif. While multiple isotypes are glycosylated in the Fc, we will focus on glycosylation in the context of the four IgG subclasses (Vidarsson et al. 2014).

In the past 20 years, the role of antibody glycosylation as an important parameter modulating the potency of effector functions has been firmly established through advances in monoclonal antibody research and development, as well as in studies of natural immune responses in the context of infectious and autoimmune disease. Here we focus on recent research considering the glycosylation of antigen-specific antibodies in these settings.

18.5 Importance of Ab Glycosylation

The Fc domain contains a consensus N-linked glycosylation site that is typically occupied by a heptasaccharide core structure consisting of four N-acetylglucosamine (GlcNAc) and three mannose moieties that form a biantennary complex (Liu 2015). Additional glycosylation features such as fucose, galactose, sialic acid, and GlcNAc can be added later to the core structure to produce over 30 distinct glycovariants. As both heavy chains are glycosylated, a single IgG molecule can have a diverse array of glycosylation heterogeneity (Jefferis 2009). Nuclear magnetic resonance (NMR) studies have shown that variability in the glycans at this conserved position has a profound effect on the hinge region conformation (Yamaguchi et al. 2006). Similarly, interactions with Fcγ and other IgG and glycan receptors are entirely dependent on or modified by glycan composition and conformation, thus the type of glycan occupying this site modifies antibody effector function (Saunders and Conceptual 2019). Unlike genetically templated factors that impact IgG activity, such as Fv sequence and Fc subclass, antibody glycosylation is remarkably varied, resulting in a high level of microheterogeneity that facilitates the fine tuning of antibody function (Alter et al. 2018a). These dynamic changes in antibody glycosylation can have a subtle or profound effect in their interactions or downstream functions.

18.6 Typical Serum IgG Fc Glycan Composition

Given the importance of IgG Fc glycans, the composition of serum antibodies has been evaluated in a number of populations, providing insight into changes associated with age, sex, hormone levels, and disease status. Nonetheless, "typical" compositions have been articulated among healthy individuals (Fig. 18.1), and deviations from this profile suggest active processes regulating this posttranslational modification at multiple levels.

Serum IgG Fc is typically overwhelmingly fucosylated (>90%) (Gudelj et al. 2018). However, skewed glycosylation variants, produced by chemoenzymatic modifications or expressed in engineered cells, have been produced that lack this fucose moiety, and as a result exhibit significantly improved effector function. For example, an afucosylated form of an anti-CD20 IgG1 showed a 50-fold improvement in binding to FcγRIIIa and enhanced ADCC activity (Shields et al. 2002). Later, structural studies found that the fucose on the Fc glycan clashes with a $GlcNAc_2$ group of an FcγRIIIa glycan, thereby providing a structural rationale to the improved ADCC activity of afucosylated antibody (Ferrara et al. 2011).

About 10% of all circulating IgGs in healthy human adults exhibit bisected Fc glycans (Gudelj et al. 2018), which have been shown previously to relate to ADCC activity (Hodoniczky et al. 2005). However, this amplification in ADCC, caused by the increased engagement of the FcγRIII, is believed predominantly to be due to the indirect role of bisection in decreasing fucosylation, rather than a direct consequence of its presence in the antibody structure (Shinkawa et al. 2002).

Similarly, agalactosylated, monogalactosylated, and digalactosylated glycan structures account for approximately 35%, 35%, and 15% of circulating IgG Fc-glycans, respectively (Gudelj et al. 2018). A prominent bias towards agalactosylated antibodies has been observed in people with active autoimmune and inflammatory diseases (Parekh et al. 1989; Tomana et al. 1992; Rademacher et al. 1994; Decker et al. 2016), however, a clear consensus on cause or consequence is yet to be achieved (Alter et al. 2018a). Furthermore, there are conflicting reports on the role of galactosylated antibodies in mediating proinflammatory activities, with some reports observing the presence of galactosylation on the IgGs to enhance the ADCC and complement binding (C1q) in vitro (Nimmerjahn et al. 2007; Peschke et al. 2017; Thomann et al. 2015; Tsuchiya et al. 1989), while others have noted a dampening of an inflammatory response by highly galactosylated immune complexes (Karsten et al. 2012). A lack of correlation between the presence or absence of galactosylation on IgGs and corresponding in vivo activity has also been reported (Nimmerjahn et al. 2007), suggesting that the consequences of variable galactosylation may be best investigated per disease model and per antibody.

Lastly, approximately 10% of circulating IgG Fc is sialylated (Gudelj et al. 2018). Sialylated IgG Fc is associated with an anti-inflammatory profile of antibodies in mouse models, in which neuraminidase-treated, asialylated pooled human IgG (IVIg) has been observed to abrogate the normally anti-inflammatory activity of IVIg (Kaneko et al. 2006). However, this mechanism of action remains controversial

in humans. Discrepant observations have been made as to the ability of IgG to interact with the candidate receptor proposed on the basis of mouse studies (Anthony et al. 2008; Temming et al. 2019), and sialylated IgG has shown slightly elevated binding to activating FcγR and C1q, and associated effector functions (Dekkers et al. 2017; Subedi and Barb 2016), which would suggest a greater inflammatory capacity.

18.7 Variations in Ab Glycoprofiles

Deviations from these "typical" profiles have been associated with diverse physiological and immunological states. For example, changes in total serum IgG Fc glycosylation are observed in early life (Cheng et al. 2019), in adolescence (Gudelj et al. 2018; de Haan et al. 2016), and in association with hormonal status (Ercan et al. 2017), as well as more gradual changes during immune senescence (Krištić et al. 2013), across a broad range of glycoforms and constituent sugar moieties. In the context of ongoing inflammation, such as observed in chronic infection (Moore et al. 2005) or autoimmunity (Parekh et al. 1985), global IgG Fc glycosylation is often modified, showing reduced galactose and sialic acid content (Lastra et al. 2009).

Beyond approaches to evaluate these global changes, the role of Fc glycans in antibody function has also motivated the development of robust methods to define the glycosylation profiles of antigen-specific antibodies (ASA) purified from serum. Early questions about ASA fractions related to whether they are typically composed of IgG Fc glycovariants with similar prevalence to those observed for total serum IgG, and if not, whether glycoprofiles vary by pathogen, antigen, and epitope specificity.

18.8 ASA Glycosylation in Infectious Disease

In the context of responses to the HIV envelope protein among chronically infected individuals, HIV envelope glycoprotein-specific antibodies were found to exhibit reduced galactosylation, fucosylation, and sialylation (Ackerman et al. 2013), even when compared to global serum IgG Fc glycan profiles that were shifted in these same directions as compared to uninfected and acutely infected individuals (Moore et al. 2005). Among ASA, galactosylation levels correlated with Ab-dependent inhibition of viral infection and replication and were consistent with glycosyltransferase and glycosidase expression in peripheral B cells (Ackerman et al. 2013). Perhaps surprisingly, these global and HIV-specific plasma IgG Fc glycan changes were not resolved by either antiretroviral drug therapy or in the context of spontaneous virus control. Subsequent studies have shown the contribution of HIV-specific IgG glycans to predicting HIV-specific antibody effector functions (Alter et al. 2018b) and vaccine efficacy (Vaccari et al. 2016; Ackerman et al. 2018).

These and other early studies have firmly established that ASA can differ from total serum IgG in their glycosylation states. As methods for analysis of ASA have advanced, analysis of ASA targeting different proteins has become increasingly feasible but not yet common. To the extent studies have addressed multiple target antigens, there has been some evidence for consistent glycoforms across distinct specificities and other cases in which different antigen-specificities, or even different epitope-specificities within the same protein have shown distinct profiles. For example, in tuberculosis, distinct ASA IgG Fc glycan profiles for two different antigen types were reported to show similar glycan profiles to each other, but with striking decreases in fucose and increases in galactose, sialic acid, and bisecting GlcNac as compared to total serum IgG Fc (Lu et al. 2020). In contrast, Wang et al. reported that the abundance of sialylation and fucosylation among influenza hemagglutinin-specific (HA) IgG differed depending on specificity of the Fab domain. Antibodies to the HA globular head were significantly more sialylated and fucosylated than those directed against the HA stem domain (Wang and Ravetch 2019), though it may be important to keep in mind that the globular head functions as a sialic acid-binding protein.

One of the most interesting examples of the effect of ASA Fc glycosylation comes from the setting of flavivirus infection. This family of viruses has been associated with a phenomenon called Antibody-Dependent Enhancement (ADE), in which virus-specific antibodies increase infection of FcγR-bearing target cells. Among these, dengue is a mosquito-borne pathogen caused by four distinct but closely related dengue virus (DENV) types. Recovery from infection is believed to typically provide immunity against infection from the same type. However, cross-type immunity is partial and temporary. Subsequent (secondary) infection by another serotype is associated with an increased risk of developing severe dengue via ADE (Katzelnick et al. 2017; Guzman et al. 2013). While prior work has shown that waning antibody titer is associated with severe disease upon secondary exposure (Katzelnick et al. 2017), recent work has highlighted the potential importance and clinical impact of the glycosylation of dengue-specific antibodies. As perhaps the most elegant setting in which to evaluate ADE, severe disease of neonates is associated with the level of passively transferred maternal dengue-specific antibody that is afucosylated, resulting in dengue hemorrhagic fever or dengue shock syndrome (Wang et al. 2017; Thulin et al. 2020; Khandia et al. 2018). This potent ADE response is thought to manifest via non-neutralizing, dengue-specific antibodies that exhibit increased affinity to the activating FcγRIIIA receptor.

In the context of coronavirus disease 2019 (COVID-19), Fc glycans of IgG antibodies to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) envelope spike and nucleocapsid proteins differ from those of total serum IgG (Larsen et al. 2020). In this study, these profiles were observed to differ between spike and capsid, and multiple studies have observed that spike-specific IgG Fc afucosylation is correlated to disease severity (Larsen et al. 2020; Chakraborty et al. 2020), with some evidence that they may contribute to pathology via inducing inflammatory responses from macrophages (Hoepel et al. 2020). Global serum IgG glycans have also been reported to diverge according to COVID-19 severity, with

decreased bisecting GlcNAc observed in multiple cohorts (Petrovic et al. 2020). The role of IgG Fc glycosylation of ASA remains to be defined in many more infectious disease settings. Like in COVID-19, HIV, and other settings in which ASAs have been profiled, intriguing observations regarding differences in global IgG Fc glycosylation abound—such as in meningococcal sepsis (Haan 2018), visceral leishmaniasis (Haan 2018; Gardinassi et al. 2014), and tuberculosis (Lu et al. 2016, 2020)—and have been found to relate to disease status or outcomes.

18.9 ASA Glycosylation in Allo/Autoimmunity

Rheumatoid arthritis (RA) is a common systemic inflammatory autoimmune disease in which joint synovium is affected by a dysregulated immune system. RA is typically associated with serological evidence of systemic autoimmunity as indicated by the presence of autoantibodies in serum and synovial fluid (Coutant 2019; Song and Kang 2010). Instead of being characterized by specific reactivity to a particular autoantigen, RA is associated with antibodies reactive against a wide spectrum of autoantigens, which can make the etiology of disease progression in RA patients very different. Among various autoantigens targeted in RA, anti-citrullinated protein antibodies (ACPA) have been identified as a useful marker in diagnosis (Coutant 2019) and predicting whether undifferentiated arthritis will progress to RA (Forslind et al. 2004). ACPA are associated with an increased risk of developing bone erosions (Rönnelid et al. 2005; Rycke et al. 2004), suggesting their potential to contribute to joint pathology. Like total serum IgG, long known to show decreased galactosylation, ACPA are observed to exhibit further reduction in sialylation and galactosylation (Scherer et al. 2010; Ohmi et al. 2016), though there is some evidence that the IgG subclasses may differ from each other in this regard (Lundström et al. 2014). Reinforcing the controversy regarding the potentially conflicting roles of sialylated IgG in different species, but supporting the role of glycoengineered ASA as therapeutic interventions, sialylated ACPA have been shown to reduce arthritis pathology in a mouse model (Ohmi et al. 2016).

Whether ACPA are a cause or consequence of RA status remains controversial, but they have been reported to activate effector cells via FcγR (Clavel et al. 2008), whose allotypic and copy number variation have sometimes but not always been observed to associate with RA status and severity (Thabet et al. 2009; Kastbom and Ahmadi 2005; Nieto et al. 2000; Radstake et al. 2003). Further, several longitudinal studies have observed that galactosylation and sialylation levels of ACPAs decreased shortly before symptom onset in patients who had ACPA but no evidence of RA at baseline (Pfeifle et al. 2017; Harre et al. 2015; Rombouts et al. 2015), suggesting the potential value of measuring the level of ACPA galactosylation/sialylation as a biomarker to predict the risk of progression from pre-clinical disease to chronic inflammatory disease. Beyond differences between ACPA and total IgG Fc glycosylation, differences in ACPA Fc glycan profiles have also been noted between individuals with and without rheumatoid factor, and between serum and

synovial fluid (Scherer et al. 2010). While some have interpreted these differences to potentially relate to active alteration of Fc glycans in affected joints, the lack of differences in total serum and synovial fluid IgG1 agalactosylation suggests that alternative mechanisms may be at play. To this end, ACPA-secreting plasma cells have been reported to exist in synovial fluid (Rodríguez-Bayona et al. 2007), suggesting the possibility that differences in systemic versus synovial ACPA Fc glycosylation may be driven by differences associated with plasma cells in the synovium and elsewhere.

Beyond these alterations in Fc glycosylation, ACPA have more recently been reported to exhibit striking glycosylation of their Fab domains. Unlike total IgG, a majority of ACPA variable domains are glycosylated (Lloyd et al. 2018; Hafkenscheid et al. 2019; Hafkenscheid et al. 2017). Unlike their Fc domains, these APCA Fab glycans are overwhelmingly sialylated (Hafkenscheid et al. 2017). Variable domain glycosylation has also been reported to modify antigen binding among ACPA (Rombouts et al. 2016), suggesting the potential for antibody glycosylation in both variable and crystallizable domains to contribute to RA pathogenesis.

Functional consequences of variations in the profile of ASA have also been reported in fetal or neonatal allo-immune thrombocytopenia (FNAIT). In this disease condition, fetal allo-antigens induce production of maternal antibodies that are then transported across the placenta and drive lysis of fetal cells. While allotypic variation of a variety of maternal fetal antigens is possible, the best studied is that of rhesus D (RhD) antigen incompatibility. Curiously, this incompatibility, which resulted in hemolytic disease in 1% of babies born through the 1940s, 40% of which would die as a result (Bowman 2003), is treated by administration of IVIG from RhD-sensitized donors. While like IVIG used in other indications, the precise mechanisms of this intervention remain unclear; prevention of sensitization, immunomodulatory effects, and accelerated clearance of endogenous maternal IgG have all been proposed as candidate mediators. To this end, the RhD-specific antibodies in at least one commercial product show increased galactosylation and sialylation relative to the entire mixture of antibodies in that product (Winkler et al. 2013), suggesting their potential immunosuppressive character. Evaluations of the mechanism of action have been hampered by the difficulty in recapitulating protective effects of polyclonal RhD IgG with monoclonal antibodies. The difference in the effect of polyclonal versus monoclonal antibody infusions may relate to differential glycosylation of RhD-specific fraction or entire pool, differences in affinity and avidity, altered red blood cell clearance capacity, or other factors, but have led to observations of alternatively enhanced or inhibited maternal sensitization, leaving many unanswered questions (Kumpel 2007; Kumpel et al. 1995). To this end, it has been recently reported that RhD-specific monoclonal antibodies varied in their ability to clear RhD+ target cells and prevent alloimmunization, dependent on their fucosylation status and associated ADCC activity (Kumpel et al. 2020). Similarly, in the context of seropositive mothers, IgG Fc fucosylation of RhD-specific antibodies have been found to correlate with ADCC activity and low fetal neonatal hemoglobin levels (Kapur et al. 2014a).

Despite questions as to mechanism, RhD+ serum IgG has all but eliminated pregnancy loss and neonatal death from RhD incompatibility in much of the world. In contrast, other less frequently observed incompatibilities have no effective preventative interventions. For a number of these antigens, maternal antibody titer is a poor indicator of pathology, and in some of these settings, variation in ASA-Fc glycosylation has been investigated for its predictive value. Here, more mixed results as to the importance of glycosylation profiles of fetal antigen-specific antibodies have been observed. As compared to RhD-specific antibodies, those recognizing red blood cell antigens K, c, and E were less distinct from total plasma IgG Fc glycans than those recognizing RhD, but nonetheless, afucosylation of Kell (K)-specific antibodies and high galactosylation and sialylation of anti-c antibodies were correlated with severe anemia of the fetus (Sonneveld et al. 2016a). In a small follow up study of maternal K-specific antibodies, IgG1 and IgG3 fractions were shown to exhibit similar glycoform prevalences, and while the previously observed relationship between afucosylation and disease severity did not meet an arbitrary significance threshold of $p = 0.05$, galactose content was shown to correlate with disease severity (Sonneveld et al. 2018).

Beyond red blood cell alloantigens, Fc glycoforms of human platelet antigen 1a (HPA-1a)-specific antibodies have been analyzed. Like other maternal alloantibody responses, HPA-1a-specific antibodies show markedly decreased levels of fucosylation as compared to total serum IgG1 (Kapur et al. 2014b). These significantly less fucosylated anti-HPA-1a antibodies showed enhanced phagocytosis of platelets on account of higher binding affinity to FcγRIIIa and FcγRIIIb, but not to FcγRIIa, compared with antibodies with a high amount of Fc fucose. Most critically, the extent of HPA-1a-specific antibody Fc fucosylation was shown to correlate with clinical disease severity. In a follow-up study, stability of ASA Fc glycans was defined and correlations between bleeding severity and fucose, galactose, and antibody titer were observed (Sonneveld et al. 2016b). Similarly, Jo1 anti-histidyl tRNA synthetase autoantibodies, which are observed in idiopathic inflammatory myopathy and anti-synthetase syndrome, have demonstrated similar reductions in galactose, sialic acid, and fucose, with glycoprofiles relating to disease status (Fernandes-Cerqueira et al. 2018).

Collectively, auto- and alloimmune responses have supported the importance of Fc glycans of ASA to diverse antigens. These observations have motivated investigation of deglycosylated IgGs to prevent FNAIT (Bakchoul et al. 2013), and sialylated ACPA to treat RA (Ohmi et al. 2016). While similar evaluation of alloantibodies in the setting of organ transplant has proven challenging, the role of effector functions is well established, with assessment of complement deposition associated with transplant- or donor-specific antibodies (DSA) forming part of the basis for evaluation of suitability of transplant (Zeevi et al. 2013; Mohan et al. 2012; Stegall et al. 2011; Lefaucheur et al. 2010), and enzymatic Fc restriction of serum IgG showing potential in reducing transplant loss associated with DSA positive organ recipients (Jordan et al. 2017).

18.10 In vivo Fc Glycan Programming

The importance of IgG Fc glycans to Ab biology in vivo has motivated a number of interventions that take advantage of this dependence. Beyond glycoengineering of therapeutic antibodies to optimize their activity, sophisticated new approaches are being explored to control antibody activity. These include leveraging B cell-independent sialylation (Jones et al. 2016) by administration of exogenous galactosyl and sialyltransferase in order to accomplish in vivo sialylation and thereby ameliorate autoimmune disease (Pagan et al. 2018). Similarly, changes in sialyltransferase expression induced by estrogen therapy suggest alternatives to exogenous enzyme therapy (Engdahl et al. 2018).

As opposed to extending IgG Fc glycans, glycan restriction is also being employed toward the same goal of reducing autoimmunity. Glycosidase therapy, most notably EndoS from *S. pyogenes*, the same organism that expresses the IgG protease IdeS used to disarm HLA alloantibodies in kidney transplant, has been investigated in diverse autoimmune conditions in animal models. These settings include IgG-driven thrombocytopenia purpura (Collin et al. 2008), collagen autoimmunity (Hirose et al. 2012), anti-neutrophil cytoplasmic autoantibody-mediated glomerulonephritis (van Timmeren et al. 2010), and autoimmune hemolysis (Allhorn et al. 2010). Challenges to clinical translation remain, including the consequences of globally eliminating effector function non-specifically, as well as the induction of anti-enzyme antibodies, but recent translation of the Fc protease IdeS suggests that these barriers may be surmountable (Collin and Bjorck 2017).

Other possibilities, such as the ability to vaccinate to drive specific inflammatory or anti-inflammatory antibody responses, also exist. A future in which allergen therapy leverages B cell transcriptional programs to not only undergo CSR toward less inflammatory IgG4 molecules but also toward anti-inflammatory glycans comes to mind, as has been shown to lessen allergic reactions in a mouse model using a recombinant glycoengineered antibody (Epp et al. 2017). To this end, Vestrheim et al. considered four distinct bacterial and viral vaccines and observed that the IgG subclass that dominated the response exhibited a temporal increase in galactosylation and sialylation for most vaccinees (Vestrheim et al. 2014). Other studies have observed this effect only within the ASA fraction (Selman et al. 2012).

With a more nuanced perspective, Larsen et al. compared and contrasted ASA targeting enveloped and non-enveloped viral pathogens and found decreased fucose content that is consistent with responses to infection by enveloped viruses, though to varying extents (Larsen et al. 2020). Natural infection, at least in the case of Hepatitis B Virus, was found to better induce afucosylated IgG1 as compared to immunization with a protein subunit vaccine. In contrast, attenuated Mumps virus vaccination induced a similar level of IgG1 afucosylation as natural infection. A study considering HIV-specific IgG Fc glycans observed that vaccination was able to overcome the normally observed variations in total serum IgG associated with geography (Mahan et al. 2016). ASA showed similar glycosylation patterns for a given vaccine, but distinct vaccine regimens resulted in distinct ASA glycosylation profiles.

These and complementary observations related to difference in induction of the IgG subclasses mediated by distinct antigen, pathogen, or vaccine stimuli suggest the existence of "rules" regulating the CSR and glycosylation processes in B cells. While refined insight into these pathways continues to develop, using an in vitro B-cell culture system resembling the in vivo T-cell-dependent antibody production, Wang et al. showed that B-cells secreted variably glycosylated IgG1 when stimulated with TLR ligands, metabolites, and cytokines (Wang et al. 2011). Indeed, because the antibody Fc domain itself can regulate responses by antigen-presenting cells and B-cells, manipulation of Fc glycans in the context of immune complex vaccines has been used to intentionally influence subsequent Ab induction/maturation (Lofano et al. 2018).

18.11 Summary and Future Perspectives

Distinctly different global IgG and ASA Fc profiles have been observed in both infectious disease and auto- and alloimmune settings. Studying the Fc glycosylation profile of ASA presents an excellent opportunity to understand the mechanistic underpinnings and the in vivo regulation of the diverse adaptive immune processes that define protective and pathological humoral responses. To this end, many unanswered questions remain.

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Compliance with Ethical Standards

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Conflict of Interest PB declares he has no conflict of interest. MEA declares that she has no conflict of interest.

Ethical Approval This chapter does not contain any studies with human participants or animals performed by any of the authors.

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Correction to: Antibody Glycosylation

Marija Pezer

Correction to:
M. Pezer (ed.), *Antibody Glycosylation*,
Experientia Supplementum 112,
https://doi.org/10.1007/978-3-030-76912-3

The original version of Chapters 4 and 15 was inadvertently published with few mistakes, and these chapters are updated with the following corrections.

Chapter 4:

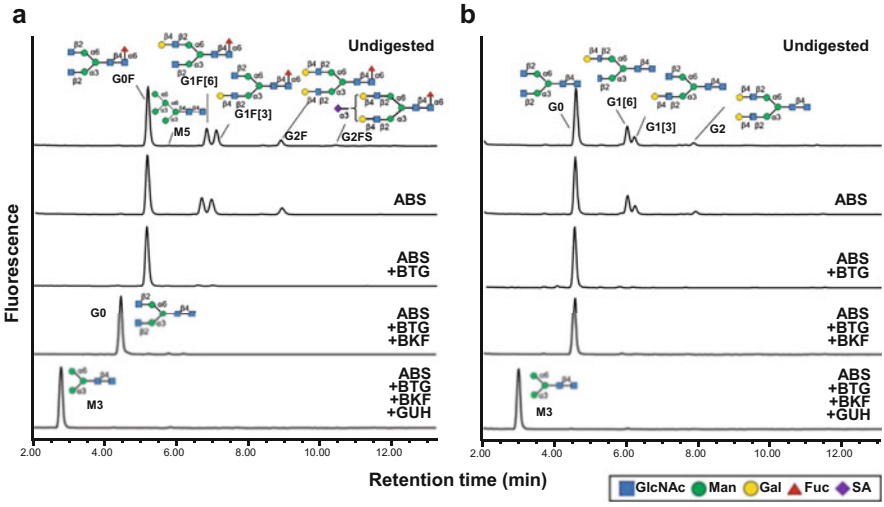
- On page 137, the sentence "Samanta Cajic and René Hennig contributed equally with all other contributors" was changed to "Samanta Cajic and René Hennig contributed equally."
- On page 142, Fig. 4.1 was updated with the correct figure provided in the manuscript, which has all the stars in green.
- On pages 144 and 145, Figs. 4.2 and 4.3 were exchanged. The legends are correct.
- On page 145, Fig. 4.3 is replaced with new figure since color and text were overlapping.
- On page 160, Fig. 4.6 was replaced with the one provided in the manuscript since few texts inside the figure are incorrect.
- Figures 4.2 and 4.3 were incorrectly placed on pages 144 and 145, respectively, whereas the citations were provided on pages 153 and 154. This was corrected by placing the figures near the citations.

The updated online versions of the chapters can be found at
https://doi.org/10.1007/978-3-030-76912-3_4,
https://doi.org/10.1007/978-3-030-76912-3_15

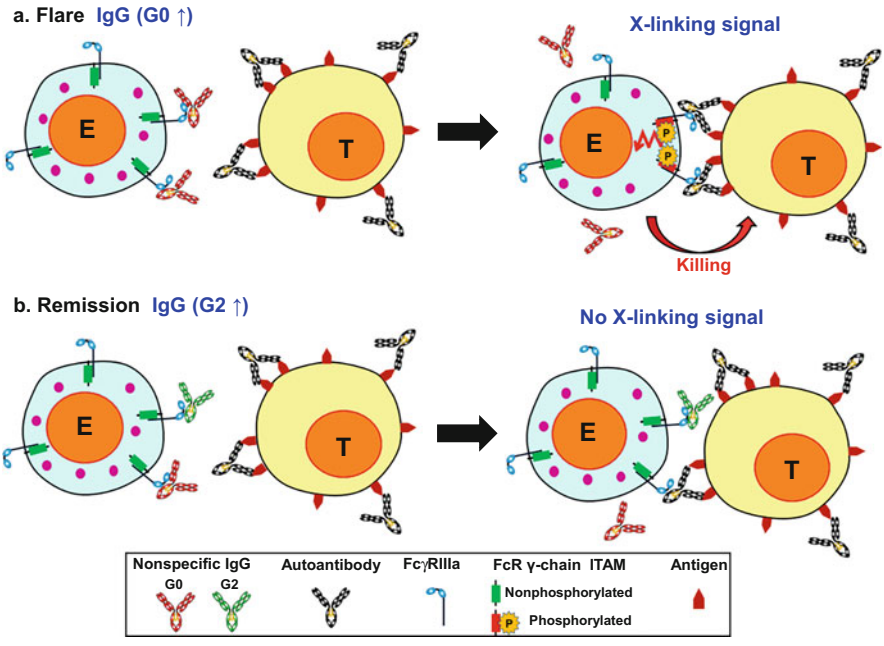
M. Pezer (ed.), *Antibody Glycosylation*, Experientia Supplementum 112,
https://doi.org/10.1007/978-3-030-76912-3_19

Chapter 15:

- The second affiliation of fourth author Dr. Pauline M. Rudd is updated as Bioprocessing Technology Institute, Agency for Science, Technology and Research, Centros, Singapore.
- On page 484, part labels (a) and (b) were inserted in Fig. 15.1.
- On page 484, Fig. 15.1 was replaced with a revised one:



- On page 495, Fig. 15.6 was replaced with the revised one:



- In page 503, line 40, Mimura, Y. et al., manuscript in preparation has been updated as Mimura et al. 2022 and the corresponding reference has been included in page 512.