

## Review

# Monitoring of immunoglobulin *N*- and *O*-glycosylation in health and disease

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## Abstract

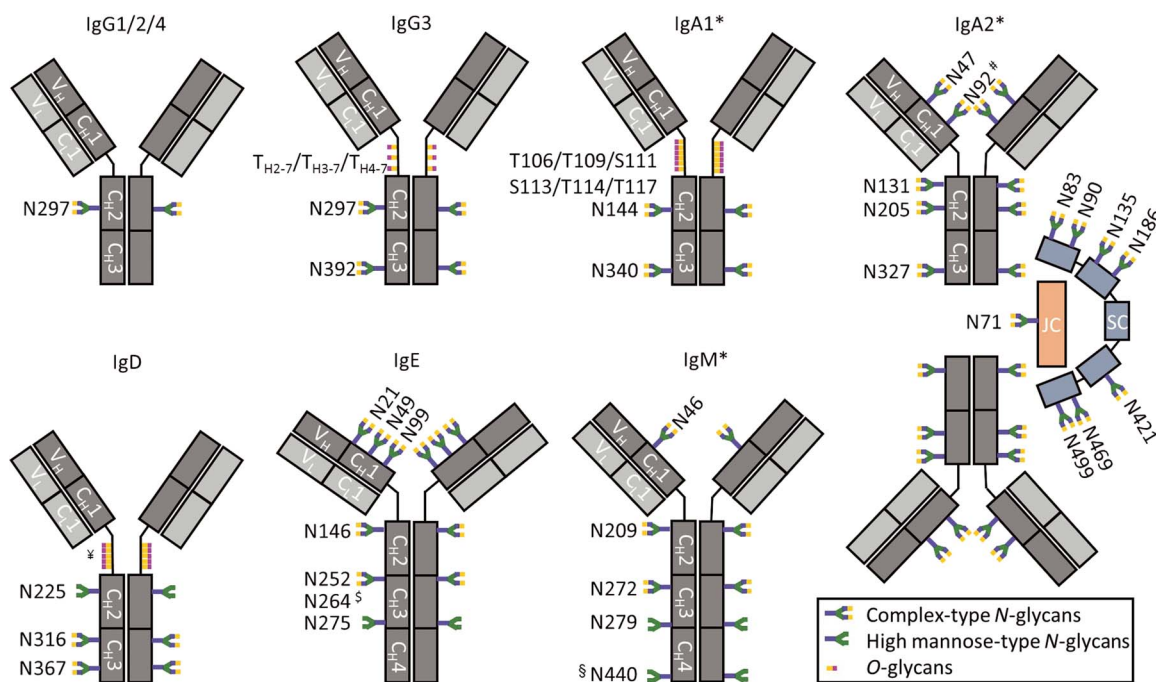
Protein *N*- and *O*-glycosylation are well known co- and post-translational modifications of immunoglobulins. Antibody glycosylation on the Fab and Fc portion is known to influence antigen binding and effector functions, respectively. To study associations between antibody glycosylation profiles and (patho) physiological states as well as antibody functionality, advanced technologies and methods are required. In-depth structural characterization of antibody glycosylation usually relies on the separation and tandem mass spectrometric (MS) analysis of released glycans. Protein- and site-specific information, on the other hand, may be obtained by the MS analysis of glycopeptides. With the development of high-resolution mass spectrometers, antibody glycosylation analysis at the intact or middle-up level has gained more interest, providing an integrated view of different post-translational modifications (including glycosylation). Alongside the in-depth methods, there is also great interest in robust, high-throughput techniques for routine glycosylation profiling in biopharma and clinical laboratories. With an emphasis on IgG Fc glycosylation, several highly robust separation-based techniques are employed for this purpose. In this review, we describe recent advances in MS methods, separation techniques and orthogonal approaches for the characterization of immunoglobulin glycosylation in different settings. We put emphasis on the current status and expected developments of antibody glycosylation analysis in biomedical, biopharmaceutical and clinical research.

**Key words:** antibody, biopharmaceutical, glycan, glycoproteomics, mass spectrometry

## Introduction

The diverse roles of glycosylation in various biological and pathological processes as well as the importance of protein glycosylation in the development of biopharmaceuticals have, over the past decade, received broad appreciation in the life sciences (Walt et al. 2012). The best studied glycoproteins in terms of the structure and function of their glycosylation are immunoglobulins (Figure 1), in particular human immunoglobulin G (IgG; Arnold et al. 2007; Dekkers et al. 2017) which features mostly complex biantennary glycans with varying degrees of galactosylation, sialylation, bisection and core fucosylation. Since the 1980s a vast body of literature has become

available, detailing immunoglobulin glycosylation features across the different antibody isotypes. Additionally, these data increasingly describe immunoglobulin glycosylation in a subclass-, allotype- and site-specific manner (Huhn et al. 2009; Zauner et al. 2013; Plomp et al. 2016). Importantly, antibody glycosylation has been shown repeatedly to differ between sexes and with age as well as with various environmental and life-style factors such as urbanization and smoking (Gudelj et al. 2018). Moreover, antibody glycosylation has been found to be skewed in numerous diseases including various autoimmune diseases, infectious diseases and different types of cancer (Parekh et al. 1985; Ackerman et al. 2013; Gudelj et al. 2018). These



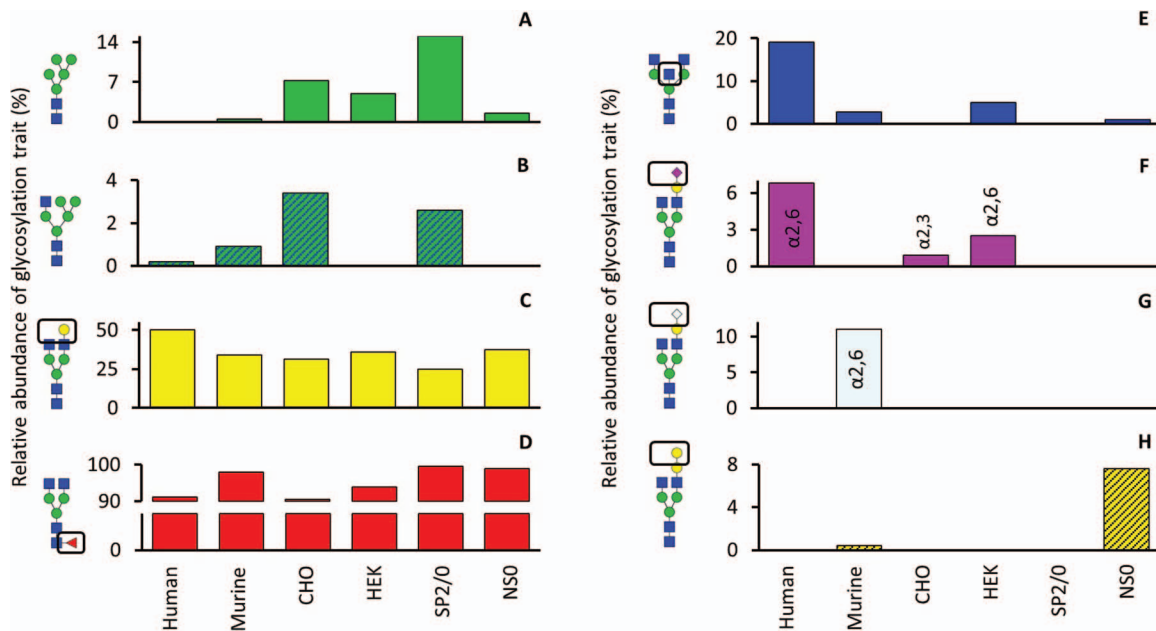
**Fig. 1.** Schematic representation of the glycosylation sites of IgG, IgA, IgD, IgE and IgM. The IgG glycosylation sites are indicated by their amino acid number according to literature, e.g. [Arnold et al. 2007](#) and [Plomp et al. 2015](#), while the other isotypes follow UniProt numbering (for an overview of Ig glycosylation site nomenclature, see [Plomp et al. 2016](#)). Each Ig monomer is composed of two heavy chains (dark gray) and two light chains (light gray), connected by disulfide bonds. The chains are further subdivided as constant (C) and variable (V) domains. Polyclonal immunoglobulins may carry additional occupied glycosylation sites in their (hyper) variable regions in the Fab domain, which are not indicated in this figure. \*IgA2 is shown in its dimeric form and in complex with the joining chain (JC; red) and the secretory component (SC; blue; present only for secretory IgA), both linked via disulfide bonds. Although not shown here, also IgA1 can form dimers, while IgM generally forms pentamers, both in combination with one joining chain. #N92 on IgA2 is only incorporated in an *N*-glycosylation consensus sequence in the IgA<sub>n</sub> and IgA2m(2) allotypes; N92 in IgA2m(1) is not glycosylated ([Plomp et al. 2018](#); [Chandler et al. 2019](#)). §Although N264 of IgE is part of an *N*-glycosylation consensus sequence (NHS), this site was reported to be non-glycosylated when studied at the glycopeptide level ([Plomp et al. 2014](#); [Wu et al. 2016](#)). ¶There are nine potential *O*-glycosylation sites present in the hinge region of IgD (S109, S10, T113, S121, T126, T127, T131, T131 and T135) for which different occupancies have been reported ([Takayasu et al. 1982](#); [Mellis and Baenziger 1983](#)). §IgM N440 was reported to have a 30–40% site occupancy when derived from human plasma ([Chandler et al. 2019](#)). The green–blue–yellow structures at the glycosylation sites represent the presence of mainly complex type *N*-glycans, while the green–blue structures represent the presence of mainly high-mannose type *N*-glycans. The yellow–pink structures indicate the presence of *O*-glycosylation.

findings mainly concerned IgG glycosylation, but recently IgA glycosylation has also attracted attention and was found to be associated with rheumatoid arthritis as well as pregnancy ([Bondt et al. 2017](#)). In accordance with the IgG glycosylation changes observed in health and disease, multiple associations of this antibody glycosylation have been shown with inflammatory markers such as cytokines and C-reactive protein ([Plomp et al. 2017](#)). Likewise, associations of IgG glycosylation with markers of metabolic health, such as blood glucose, lipoprotein particles and central adiposity, have been established ([Lemmers et al. 2017](#); [Plomp et al. 2017](#); [Russell et al. 2019](#)). Next to development, environment, inflammation and metabolism, a strong genetic and also epigenetic influence on antibody glycosylation has been revealed ([Lauc et al. 2010, 2013, 2014](#); [Klasic et al. 2016, 2018](#)).

While our knowledge of human antibody glycosylation has been growing considerably, also the immunoglobulin glycomes of important model systems become increasingly mapped. These include the IgG glycosylation of cell lines, such as CHO, HEK, NS0 and SP2 cells, and various murine strains ([Figure 2](#); [Stadlmann et al. 2008](#); [Montesino et al. 2012](#); [Dekkers et al. 2016](#); [de Haan et al. 2017](#); [Goh and Ng 2018](#)) and IgA glycosylation in mammalian expression systems as well as glyco-engineered plants ([Dicker et al. 2016](#); [Rouwendal et al. 2016](#)). It is crucial for the proper translation of

research results between different systems to take the source-specific glycosylation features of antibodies into account.

Our understanding of the role and diversity of protein glycosylation in general, and antibody glycosylation in particular, has grown in parallel with the development and implementation of new molecular and analytical tools in both biomedical and biopharmaceutical research. The field of glycoengineering has been instrumental for furthering our glycobiological understanding. Prominent developments in this area include (1) molecular biology tools to edit the glycoenzyme makeup of eukaryotic cells, (2) *in vitro* tools such as small molecule inhibitors and medium additives to modulate protein glycosylation during cell culture and (3) the availability and application of glycosyltransferases and glycosidases for the post-cell culture editing of protein glycosylation ([Dekkers et al. 2016](#); [Giddens et al. 2018](#); [Gupta and Shukla 2018](#)). Of equal importance has been the development of analytical tools to aid in the study of both structure and function of specific glycans and glycoforms. Here, released glycan analysis by hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLU) has a central role for the analysis of antibody glycosylation in both biomedical and biopharmaceutical settings. HILIC-FLU methods often serve as a gold standard for the characterization of antibody glycosylation, featuring excellent



**Fig. 2.** IgG Fc glycosylation is dependent on the expression system. Shown are the relative abundances of (A) high mannose-type glycans, (B) hybrid-type glycans, (C) galactosylation, (D) fucosylation, (E) the presence of a bisecting GlcNAc, (F) Neu5Ac sialylation, (G) Neu5Gc sialylation and (H)  $\alpha$ 1,3 galactosylation of human IgG1 (Plomp et al. 2017; Simurina et al. 2018), murine IgG1 (de Haan et al. 2017), Trastuzumab produced in CHO cells (Stadlmann et al. 2008), human IgG1 expressed in free style HEK cells (Dekkers et al. 2016), Cetuximab expressed in SP2/O cells and Daclizumab expressed in NSO cells (Stadlmann et al. 2008). Notably, while in most systems the sialylation is  $\alpha$ 2,6-linked, in CHO cells exclusively  $\alpha$ 2,3-linked sialylation is present. Next to the expression system, also biological effects (like age and disease for human IgG glycosylation and mouse strain for murine glycosylation) and the fermentation process (for cellular expression systems) have an effect on Fc glycosylation. For example, also Neu5Gc sialylated species have been reported on IgG expressed in NSO cells (Montesino et al. 2012).

robustness, accuracy and precision (Reusch, Habberger, Maier, et al. 2015). Although this method provides sufficient resolution to profile the moderately complex global IgG glycome, it does not suffice for the in-depth characterization of site-specific antibody glycosylation in general.

Antibody glycosylation analysis has recently seen several specific developments which are pivotal for further glycobiological research. This includes the development of high-throughput techniques such as capillary gel electrophoresis with laser-induced FLU (CGE-LIF) and various mass spectrometric (MS) approaches (Szekrenyes et al. 2012; Reusch et al. 2014; Reusch, Habberger, Falck, et al. 2015; Reusch, Habberger, Maier, et al. 2015; Szigeti and Guttman 2017). Furthermore, a vast body of work was performed enabling site-specific glycosylation profiling of immunoglobulin isotypes and subclasses analyzing glycopeptides by MS (Selman et al. 2012; de Haan et al. 2015; Bondt et al. 2016; Chandler et al. 2019). Another important aspect of antibody glycosylation analysis is miniaturization in order to increase sensitivity. For example, MS methods have been instrumental for analyzing antigen-specific antibody subpopulations with high sensitivity and specificity (Wuhrer et al. 2009; Kapur et al. 2014).

Analytical technologies that focus on structural features of antibodies have been complemented by methods for studying functional aspects of antibody glycosylation. Of interest here are, for example, the scavenging functions of antibodies and the interactions between antibody Fc portions and receptors involved in effector mechanisms of the humoral immune system. This leads to the integration of structural and function analysis at the intact protein level with important roles for native-mode protein separation and native MS (Gahoual et al. 2017; Kiyoshi et al. 2018).

Here, we provide an overview of recent developments in antibody glycosylation analysis, highlighting advances in separation techniques, mass spectrometric methods and functional assays. We will describe the status, current role and expected development of antibody glycosylation analysis in biomedical research, biopharma and eventually clinical diagnostics.

## Separation-based approaches

The limited complexity of Ig glycan structures means separation-based techniques—that is without additional selectivity from MS—often provide sufficient resolution. Due to the limited overlap in glycan structures between Fc and Fab glycosylation of IgG and the occurrence of solely Fc glycosylation in many monoclonal IgG samples, their glycosylation analysis does, generally, not require a site-specific approach (Bondt et al. 2014). However, it cannot be excluded that this is different under specific (patho) physiological conditions or for Fab glycosylated monoclonal antibodies (mAbs). Therefore, it is advisable to choose a site-specific method in the initial characterization of a (potentially) Fab glycosylated IgG from unfamiliar sources, such as an understudied cell line, biofluid or pathology. Additionally, the other Ig classes have several glycosylation sites in the constant region with overlapping glycan species (Arnold et al. 2007; Plomp et al. 2014, 2018). Hence, glycopeptide analysis by MS-based methods is preferred for these in order to resolve site-specific glycosylation. HILIC-FLU remains the gold standard for released glycan analysis due to its high precision and low implementation hurdle (Reusch, Habberger, Maier, et al. 2015; Colhoun et al. 2018; Table 1). While classically 2-aminobenzamide (2-AB) or variants are

Table 1. Overview of techniques used for the analysis of immunoglobulin glycosylation

Technology	Accessibility <sup>a</sup>	Readiness <sup>b</sup>	Throughput	Glycan isomer resolution	Feature resolution	Sensitivity	Quantitation precision <sup>c</sup>	Site specific	Protein specific
Separation based									
HILIC-fluorescence of glycans	+	+	-	+	-	+/-	+	no	no
CGE-LIF	+	+	+	+	-	+	+	no	no
HILIC-fluorescence of glycopeptides	+	-	-	+	-	+/-	+	yes	no
Mass spectrometry based									
HILIC-LC-ESI-MS of glycans	+	+	-	+	+	+	+/-	no	no
PGC-LC-ESI-MS of glycans	+	-	-	+	+	+	-	no	no
CE-ESI-MS of glycans	+/-	+/-	-	+	+	+	+/-	no	no
Ion mobility-MS of glycans	+/-	-	+	+/-	+/-	+/-	+/-	no	no
MALDI-MS of glycans	+	+	+	+	+/-	+	+/-	no	no
RP-LC-ESI-MS of glycopeptides	+	+	+/-	-	+	+	+/-	yes	yes
HILIC-LC-ESI-MS of glycopeptides	+	+	-	+/-	+	+	+/-	yes	yes
PGC-LC-ESI-MS of glycopeptides	+	-	-	+	+	+	-	yes	yes
CE-ESI-MS of glycopeptides	+/-	+	-	+	+	+/-	+/-	yes	yes
MALDI-MS of glycopeptides	+	+	+	+	+/-	+	+/-	yes	yes
ESI-MS of intact antibodies	+/-	+	+	-	+/-	-	+/-	no	yes
ESI-MS of antibody fragments	+/-	+	+	-	+/-	-	+/-	no (yes for IgG)	yes
MALDI-MS of intact antibodies	-	+/-	+	-	-	-	+/-	no	yes
MALDI-MS of antibody fragments	-	+/-	+	-	-	-	+/-	no (yes for IgG)	yes
RP-LC-ESI-MS of intact antibodies	+/-	+	-	-	-	-	+/-	no	yes
RP-LC-ESI-MS of antibody fragments	+/-	+	-	-	+/-	-	+/-	no (yes for IgG)	yes
HILIC-LC-ESI-MS of intact antibodies	+/-	+/-	-	-	+/-	-	+/-	no	yes
HILIC-LC-ESI-MS of antibody fragments	+/-	+/-	-	+/-	+/-	-	+/-	no (yes for IgG)	yes
CE-ESI-MS of intact antibodies	+/-	+	-	+	+/-	+/-	+/-	no	yes
CE-ESI-MS of antibody fragments	+/-	+	-	+	+/-	+/-	+/-	no (yes for IgG)	yes
Affinity-LC-ESI-MS of intact antibodies	-	+/-	-	+	+/-	-	-	no	yes
Other approaches									
Lectins	+	+	+	+/-	-	+/-	+	no	no
Anti-carbohydrate antibodies	+	+	+	+/-	-	+/-	+	no	no
Affinity-LC-UV of intact antibodies	-	+	-	+/-	+/-	-	-	no	no
SPR array	-	+	-	+/-	+/-	-	+/-	no	no
NMR of intact antibodies	+/-	+/-	-	+	+/-	-	+	yes	yes

<sup>a</sup>The accessibility of the method indicates the hardware availability in an average laboratory.

<sup>b</sup>The readiness of the method indicates the ease of implementation of the technology for immunoglobulin glycomics, when the hardware is present.

<sup>c</sup>Precision here also takes the robustness of the precision into account.

used for fluorescence labeling (Huffman et al. 2014), procainamide and RapiFluor are becoming increasingly popular due to their improved fluorescence yield (Keser et al. 2018). HILIC-FLU separates based on number of galactoses and *N*-acetylneuraminic acids (Neu5Ac) and the absence or presence of core fucose and bisecting *N*-acetylglucosamine (GlcNAc). Additionally, galactose can be located to the  $\alpha$ 1-3 or  $\alpha$ 1-6 arm, an isomer distinction which is generally achieved with HILIC-FLU and has previously been associated, for example, with systemic lupus erythematosus (Vuckovic et al. 2015). Mixed-mode columns, combining anion exchange (AEX) resins with either HILIC or reversed-phase (RP) stationary phases, have been proposed to resolve co-elution of differentially sialylated glycans, similarly to popular weak AEX pre-fractionation (Largy et al. 2017). However, these cases are rare in state-of-the-art HILIC separations of IgG glycans (Keser et al. 2018). HILIC-FLU for glycopeptides, relying on fluorescent labeling of the peptide *N*-terminus, can be implemented as a simple add-on to well-established peptide mapping workflows (Ludger 2019). Generally, liquid chromatography (LC)-FLU approaches require at least around 30 min per analysis, limiting their throughput.

Alternatively, separations can be performed by capillary electrophoresis (CE)-LIF, using either gel-filled capillaries (CGE) or open capillaries (CZE; Borza et al. 2018; Magorivska et al. 2018; Table I). In most cases, fluorescence labeling is achieved by reductive amination with aminopyrene trisulfonate (APTS), providing three negative charges. CZE capillaries are often coated to prevent a negative influence of wall interactions on peak shape and reproducibility (Magorivska et al. 2018). CE-LIF has a high separation power, resulting in the resolution to separate all *N*-glycosylation features generally separated by HILIC-LC. This includes the distinction of sialic acid linkage isomers which may be interesting especially in the context of glycoengineering (de Haan et al. 2015). Neutralization of the sialic acids can provide an alternative selectivity between sialylated and nonsialylated glycoforms as demonstrated, for example, for the determination of hypogalactosylation in rheumatoid arthritis (Schwedler and Blanchard 2019). Migration time variation is generally managed by the implementation of standards (Szigeti and Guttman 2017). Fast CZE-LIF analysis times of 3 min have been reported (Szigeti and Guttman 2017). Alternatively, CGE-LIF provides a high-throughput platform via the multiplexing of analysis using modified DNA-analyzers and, more recently, dedicated machines with 12–96 parallel capillaries (Reusch et al. 2014; Sciex 2018). Thus, CE-LIF can be operated at high throughput, although precision seems to be slightly inferior to LC-FLU in these modes (Huffman et al. 2014; Reusch, Habberger, Maier, et al. 2015; Sciex 2018).

## Mass spectrometry-based approaches

While separation-based methods often suffice for the global analysis of IgG *N*-glycosylation, the broader and more specific monitoring of Ig glycosylation (including that of IgG) requires MS-based technologies (Table I). These methods have a large advantage over the non-MS-based methods described above, as they add an extra high-resolution dimension, based on mass-over-charge ratio ( $m/z$ ). They also provide the ability to characterize unknown glycan compositions and structures in complex samples, especially using tandem MS approaches (Reiding et al. 2018). Various complementary MS-based workflows are commonly used which, in combination, provide an in-depth overview of Ig glycosylation. These methods tend to follow one of the following approaches, namely (1) the chemical or enzymatic

release of glycans from glycoproteins, (2) the proteolytic cleavage of glycoproteins to obtain a mixture of peptides and glycopeptides or (3) the analysis of the intact glycoproteins.

## Released glycans

The analysis of released glycans provides the opportunity to perform an in-depth structural characterization of the glycoforms present in a sample, irrespective of the protein they are derived from. Inherent to this approach is the loss of protein- and site-specific information. The latter makes released glycan analysis of Igs prone to biases introduced by contaminating proteins (Lauc et al. 2018). Additionally, when glycans are released from intact Igs, no distinction can be made between glycosylation of the constant region, where glycosylation may influence the interaction with effector molecules (Gudelj et al. 2018), and the variable region, where glycosylation may impact antigen binding (van de Bovenkamp et al. 2016). On the other hand, the analysis of released glycans seems essential for the characterization of Fab-specific glycosylation of polyclonal antibodies; due to the vast heterogeneity which is often exhibited by polyclonal antibody populations, the proteomic analysis of variable and hypervariable regions is challenging, and consequently Fab glycosylation is currently studied at the released glycan level or using binding assays, rather than site-specific at the glycopeptide level (Bondt et al. 2014; van de Bovenkamp et al. 2018). Fab-specific glycan profiling is facilitated at the released glycan level when the Fab portion is separated from the Fc portion prior to glycan release. This can be obtained by using, for example, the IdeS enzyme that cleaves below the hinge region of IgG (Bondt et al. 2014). The importance of analyzing Fab glycosylation was exemplified in the case of rheumatoid arthritis, where ACPA-IgGs showed high levels of Fab glycosylation as compared to total IgG. These Fab glycosylated ACPA-IgGs may convey specific immunological roles, e.g. via binding to human lectins, and may contribute to the development of IgG-mediated autoimmunity in rheumatoid arthritis (Hafkenschied et al. 2017).

Furthermore, analysis at the released glycan level is technologically most advanced with regard to the structural elucidation of glycans by hyphenating (tandem) MS to a chromatographic or electrophoretic separation module via electrospray ionization (ESI). Powerful methods for the sensitive structural characterization of glycans in general involve porous graphitized carbon (PGC)-LC-, HILIC-LC-, CE- and ion mobility spectrometry (IMS)-MS (Gennaro et al. 2002; Mauko et al. 2012; Kolarich et al. 2015; Zhong et al. 2015; Harvey et al. 2016; Zhao et al. 2016; Keser et al. 2018). Similar to the separation-based methods, the MS-based analysis of released glycans starts with the purification of the antibody and the chemical or enzymatic release of the *N*-glycans from the proteins. Subsequently, glycans are reduced, derivatized or kept in their native form, depending on the separation and detection technique used. The choice of the sample preparation and derivatization strategy will have to be aligned with the chromatographic or electrophoretic separation technique as well as with the MS-based analysis method. Negative mode tandem MS is in particular suitable for the in-depth structural characterization of glycan isomers (Everest-Dass et al. 2013; Kolarich et al. 2015; Zhao et al. 2016), while positive mode (tandem) MS is often used for a robust and sensitive profiling (Pabst et al. 2009; Wang et al. 2017). Released glycans are relatively hydrophilic and non-sialylated species often lack readily ionizable groups, two features that hamper their efficient desolvation and ionization by ESI. These limitations are generally addressed by derivatization, e.g. by permethylation or reducing end labeling. Permethylation causes

the methylation of all carboxyl-, hydroxyl- as well as primary and secondary amine-groups of glycans, resulting in their neutralization and an overall higher hydrophobicity (Zhou et al. 2017). However, the permethylation reaction is hard to bring to completion and by-products are often observed. In addition, while permethylation comes with high-sensitivity detection, the sample preparation would need further optimization and downscaling to address limitedly available samples, as the current workflows consume microgram amounts of antibody (Shubhakar et al. 2016; Zhou et al. 2017). Alternatively, the reducing end of the *N*-glycans is employed for uniform derivatization of all species. This labeling is dependent on either the glycosylamine product that is the direct result of *N*-glycan release by PNGase F, or the reducing end aldehyde that emerges after hydrolysis of the glycosylamine. Examples of glycosylamine-dependent labels are RapiFluorMS (Lauber et al. 2015) and InstantPC (Kimzey et al. 2015), which are used for the rapid labeling of *N*-glycans in relatively pure samples. Labels that react with the reducing end aldehyde carry either an amine, aminoxy, hydrazine or hydrazide functional group. For example, the amine labels APTS (Maxwell et al. 2011), 8-aminoaphthalene-1,3,6-trisulfonic acid (Gennaro et al. 2002), procainamide (Kozak et al. 2015) and 2-AB (Pabst et al. 2009; Zhao et al. 2016) form a Schiff base with the reducing ends of the glycans, that is subsequently stabilized via a reduction step. Alternatively, aminoxy, hydrazine or hydrazide labels, such as aminoxyTMT (Zhong et al. 2015) and Girard's reagent P (Walker et al. 2011), enable rapid *N*-glycan labeling as they react with the reducing end aldehyde, but do not require a reduction step for stability. A common feature of the RapiFluorMS, InstantPC, aminoxyTMT and procainamide label is that they carry a tertiary amine, facilitating protonation for positive mode MS. This feature results in an advantage in ionization efficiency (and thus sensitivity), as compared to the amide present on the 2-AB label, enabling the analysis of released glycans from less than 5  $\mu$ g of IgG (Keser et al. 2018). In addition to the presence of a basic group, higher hydrophobicity of the labels improves ionization efficiency in ESI by a more efficient desolvation of the labeled products (Walker et al. 2011).

The separation of released glycans prior to MS analysis aids their in-depth structural characterization. HILIC-LC is a robust and broadly applied separation technique for MS-based glycan analysis, especially in the biopharmaceutical setting. An increasing number of HILIC-LC-compatible reducing end labels is reported that enable sensitive glycan analysis by positive mode MS. Prominent examples are RapiFluorMS (Lauber et al. 2015), InstantPC (Kimzey et al. 2015) and procainamide (Kozak et al. 2015). The use of RapiFluorMS labeling in combination with HILIC-LC-MS/MS recently proved to be a powerful method for the structural characterization of mAb glycosylation, identifying Gal- $\alpha$ 1,3-Gal isomers on a National Institute of Standards and Technology (NIST) reference material mAb (Hilliard et al. 2017). As compared to HILIC-LC, isomer separation by PGC-LC is even more powerful, as it separates the isomers of neutral and acidic glycoforms simultaneously. For example, *N*-glycans carrying a bisecting GlcNAc, as present on IgG and IgA, elute earlier as compared to non-bisected glycans with an extra antenna. Additionally, fucose and sialic acid isomers can be separated (Stadlmann et al. 2008; Kolarich et al. 2015; Abrahams et al. 2018). PGC-LC is commonly used in combination with negative mode tandem MS for the structural elucidation of isomeric glycans (Everest-Dass et al. 2013; Kolarich et al. 2015). A recent analysis of released glycans of IgG by TiO<sub>2</sub>-PGC-LC-MS/MS reported the presence of a high number of isomeric *N*-glycan structures, including sulfated glycoforms (Wang et al. 2017, 2018). This method

relied on the selective enrichment of acidic glycoforms on the TiO<sub>2</sub>-trap column and reported associations between a selected group of the trace glycans and rheumatoid arthritis. Notably, when in-depth analyses on minor species are performed on the level of released glycans, it is advisable to perform orthogonal proteomic as well as glycoproteomic analyses at the glycopeptide level, in order to rule out that glycomic results on minor species are confounded by minor amounts of contaminating glycoproteins (Lauc et al. 2018).

Alternative to chromatographic separations, CE efficiently separates carbohydrates based on their charge and size. Coupling CE to MS via ESI provides low-flow nano-ESI conditions and results in high sensitivity for in-depth glycomics (Lageveen-Kammeijer et al. 2019). Similarly to HILIC-LC-ESI-MS approaches, reducing end glycan labeling improves the ionization efficiency for CE-ESI-MS. Additionally, the introduction of a charged label to the neutral species allows their electrophoretic migration in CE. For example, the use of positive labels (e.g. aminoxyTMT or Girard's reagent P), in combination with either a low pH background electrolyte or the neutralization of sialylated species, facilitates normal polarity CE separation in combination with positive mode MS analysis (Zhong et al. 2015; Khatri et al. 2017). While currently not broadly used for the characterization of antibody glycosylation, CE-LC-MS does provide a promising method for the sensitive separation of isomeric glycan structures derived from immunoglobulins. This includes the possibility to multiplex the analysis, when labeling with a tandem mass tag (TMT) is performed (Zhong et al. 2015).

One of the most recent developments of *N*-glycan isomer characterization focuses on the separation of reduced *N*-glycans in the gas phase by IMS-MS. Separation by IMS-MS is based on the charge and shape of the ions, which can be converted to their collision cross section (CCS; Harvey et al. 2016, Glaskin et al. 2017). The recent publication of a library containing *N*-glycan CCSs from standard proteins, including IgG, helps to assign glycan isomers with IMS-MS (Struwe et al. 2016). However, the separation power of IMS-MS for *N*-glycans is still limited and further complicated by the existence of glycan conformers (Struwe et al. 2015). Likely, technical and computational modeling developments will enhance the power of this technique for *N*-glycan characterization in the future. In the meantime, the additional resolution of separation techniques, such as CE, can help to differentiate isomers from conformers (Jooss et al. 2018).

## Glycopeptides

In contrast to released glycan analysis, the analysis of antibody glycosylation at the glycopeptide level usually achieves significant protein specificity. Additionally, when only one site is present per peptide, this approach allows site-specific analysis. General glycopeptide-based workflows include the isolation of the antibodies, their cleavage with a proteolytic enzyme and enrichment or online separation of the glycopeptides followed by their analysis by MS (/MS). Online separation is often achieved by hyphenating LC or CE to MS via ESI (Heemskerck et al. 2013; Stavenhagen, Plomp, Wuhler, et al. 2015; Falck et al. 2017; Plomp et al. 2018; Chandler et al. 2019). Alternative methods include off-line enrichment of glycopeptides in combination with their matrix-assisted laser desorption/ionization (MALDI)-MS analysis (de Haan et al. 2015; Bondt et al. 2016; Wu et al. 2016; Bondt et al. 2017).

For the identification of glycosylation sites and the determination of site occupancy, glycopeptides are often treated by the enzyme PNGase F, which releases the *N*-glycans and converts the asparagines, to which the glycans were linked, into aspartic acids (Stavenhagen,

Plomp, Wuhrer, et al. 2015; Chandler et al. 2019). Additionally, tandem MS is used for the identification of the peptide portion of the glycopeptide and to get structural information on the glycan (Reiding et al. 2018). Lower energy collision-induced dissociation (CID) of glycopeptides results in glycan fragments, while electron-transfer dissociation (ETD) or electron-capture dissociation (ECD) provides information about the peptide sequence and the site of glycosylation. A combination of low and high energy CID (or higher-energy collisional dissociation for Orbitrap instruments) provides fragmentation information on both portions of the glycoconjugate. The general application of these techniques in glycoproteomic research, including the software packages available to interpret the acquired data, is nicely exemplified by a recent study from Stadlmann et al. (2017) and further reviewed elsewhere (Ruhaak et al. 2018).

A common stationary phase for LC separation of glycopeptides prior to MS analysis is RP-C18. This approach mainly separates based on the peptide portion of the analytes and allows the co-elution of glycosylated species with the same peptide portion (Selman et al. 2012). RP-LC was widely used over the past decades to obtain Fc-specific *N*-glycosylation profiles of the IgG subclasses after a tryptic digestion, revealing numerous associations with (patho) physiological processes, which was recently extensively reviewed (Gudelj et al. 2018). Moreover, IgG3-specific *O*-glycosylation of the hinge region was described by combining the results of trypsin and proteinase K treated IgG3 samples (Plomp et al. 2015). More recently, also the other immunoglobulin isotypes were approached in a similar manner. For example, tryptic *N*- and *O*-glycopeptides were characterized from IgA derived from plasma, colostrum and saliva, including the glycosylation sites on the J-chain and secretory component associated with secretory IgA (Deshpande et al. 2010; Plomp et al. 2018; Chandler et al. 2019). These studies revealed body fluid-specific glycosylation profiles, which indicate that careful biofluid selection is an important factor in biomarker discovery (Deshpande et al. 2010; Plomp et al. 2018). IgM purified from human plasma was recently characterized in a site-specific manner, assessing not only the different glycoforms at the five distinct *N*-glycosylation sites, but also the occupancy of these sites. While four of the sites were almost completely occupied, the C-terminal N439 showed an occupancy of only 30–40% (Chandler et al. 2019).

LC separation techniques complementary to RP-LC include HILIC- and PGC-LC. HILIC-LC is especially suitable for the separation of glycopeptides with short peptide sequences, which may not be retained by RP-LC, and it allows the efficient separation of glycosylated and non-glycosylated peptides in complex mixtures (Zauner et al. 2010). Additionally, for sialylated glycopeptides, HILIC enables the differentiation between  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids, showing  $\alpha$ 2,6-linked sialic acids to have a higher retention on a zwitterionic type-HILIC (ZIC-HILIC) stationary phase as compared to  $\alpha$ 2,3-linked sialic acids (Takegawa et al. 2006).

Similar to HILIC, PGC-LC is able to separate glycopeptides with short peptide moieties, with the added advantage that samples do not have to be loaded in high concentrations of organic solvent. However, it should be taken into account that both highly sialylated glycopeptides and glycopeptides with longer peptide sequences might be irreversibly retained on the PGC stationary phase (Stavenhagen, Kolarich, Whurer, et al. 2015). The same behavior is observed for hydrophobic matrix components, e.g. lipids or hydrophobic peptides, to a much higher degree than in RP-LC. Consequently, PGC requires (more) elaborate sample pre-purification. As the three mentioned chromatographic approaches have complementary properties, combinations are used for the characterization of immunoglobulin

glycopeptides (Liu et al. 2014; Stavenhagen, Plomp, Wuhrer, et al. 2015). For example, sequential C18-PGC-LC-MS was used to study the C<sub>H</sub>3 domain *N*-glycosylation site of IgG3, after treatment of IgG3 with trypsin and AspN. This revealed higher levels of non-fucosylated glycans at this site as compared to the conserved glycosylation site in the C<sub>H</sub>2 domain (Stavenhagen, Plomp, Wuhrer et al. 2015).

The separation mechanism of CE is complementary to RP-LC as IgG Fc glycopeptides are mainly separated based on their glycan moiety, resulting in the co-migration of identical glycoforms attached to different subclasses (Heemskerk et al. 2013). However, due to the size- and charge-based separation, CE may also be complementary to HILIC-LC when the differences between peptide portions are larger. Furthermore, as reported for released glycans, CE-ESI-MS is capable of separating *N*-glycan sialic acid isomers on the IgG glycopeptide level, showing a faster migration for  $\alpha$ 2,6-linked sialylated species as compared to  $\alpha$ 2,3-linked sialylated species (Kammeijer et al. 2017). Notably, CE-ESI-MS is an extremely sensitive technique, able to detect glycopeptides derived from IgG at low picogram amounts loaded into the capillary (Kammeijer et al. 2016). Efficient, high sensitivity sample preparation methods are now needed in order to make ultrahigh-sensitivity CE-MS available for the characterization of glycopeptides derived from, for example, the very low abundance plasma IgE.

For IgG Fc glycosylation, usually trypsin is used to digest the protein. Human IgG consists of four subclasses (IgG1–4), which have their specific biological activities as well as slightly different glycosylation. Obtaining IgG glycopeptides with peptide moieties fully specific for the IgG subclass is not trivial. While the tryptic digestion of IgG1, 2 and 4 always results in distinct peptide portions (independent of the allotypes; Table II), and can thus be separated with MS, the numerous known IgG3 allotypes vary in their peptide sequences surrounding the C<sub>H</sub>2 glycosylation site. For 2 of the 19 IgG3 allotypes full tryptic cleavage is expected to result in a peptide portion identical to the peptide portion of IgG2 (EEQFNSTFR; Table II), while most of the others are isomers of the IgG4 peptide (EEQYNSTFR; Table II) and only two of them result in a longer, unique peptide (TKPWEEQYNSTFR; Table II; Vidarsson et al. 2014). A similar situation applies for certain allotypes of the two subclasses of IgA, for which the peptide moieties surrounding the conserved glycosylation sites in the C<sub>H</sub>2 and C<sub>H</sub>3 domains are identical after tryptic digestion (Plomp et al. 2018; Table II). These challenges might be partially overcome by the use of proteases with a different specificity as compared to trypsin, as they might result in larger and more specific protein fragments. On the basis of available protein sequences and known enzyme specificities, GluC, which preferably cuts proteins C-terminal of glutamic acid and aspartic acid residues, may be a particularly promising candidate for complementing trypsin in IgG subclass- and allotype-specific glycopeptide analysis.

The in-depth site-specific glycosylation analysis of IgG3, IgA1 and IgD hinge region *O*-glycosylation remains a challenging task (Wada et al. 2010). This is due to the resistance of these heavily *O*-glycosylated regions to proteolytic digestion and the low number of cleavage sites available in these regions, often resulting in glycopeptides with multiple glycosylation sites. Recent developments in the area of *O*-glycan proteases might help to overcome these limitations, by enabling the cleavage at the *N*-terminus of *O*-glycosylated serines or threonines. The potential of such an *O*-glycan protease was recently shown for other *O*-glycosylated proteins, either used in stand-alone mode or in combination with another protease, like trypsin (Yang, Ao, et al. 2018, Yang, Onigman, et al. 2018).

**Table II.** Peptide moieties, and their masses, of the tryptic glycopeptides of human IgG and IgA

Protein	Glycosylation site Consensus numbering	UniProt	Tryptic peptide sequence (glycosylation site)	Peptide mass [M] (Da)
Human IgG1	N297	N180	EEQY <u>N</u> STYR	1188.5047
Human IgG2	N297	N176	EEQF <u>N</u> STFR	1156.5149
Human IgG3	TH2-7/TH3- 7/TH4-7	T122/T137/T152	SCD <u>T</u> PPPCPR	1071.4478
Human IgG3	N297	N227	EEQF <u>N</u> STFR <sup>a</sup>	1156.5149 <sup>a</sup>
			EEQY <u>N</u> STFR <sup>a</sup>	1172.5098 <sup>a</sup>
			TKPWEEQY <u>N</u> STFR <sup>a</sup>	1684.7845 <sup>a</sup>
Human IgG3	N392	N322	GFYPSDIAVEWESSGQPENN Y <u>N</u> TTPMMLSDGSFFLYSK	4388.9372
Human IgG4	N297	N177	EEQF <u>N</u> STYR	1172.5098
Human IgA1	T225/T228/S230/ S232/T233/T236	T106/T109/S111/ S113/T114/T117	HYTNPSQDVTVP <u>C</u> PVP <u>S</u> T <u>P</u> P <u>T</u> P <u>S</u> P <u>S</u> T <u>P</u> P <u>T</u> P <u>S</u> P <u>S</u> C <u>C</u> H <u>P</u> R	4135.8826
Human IgA1	N263	N144	LSLHRPALEDLLGSEAN <u>L</u> TCTLTGLR	2962.5909
Human IgA1	N459	N340 <sup>b</sup>	LAGKP <u>T</u> HV <u>N</u> VS <u>V</u> MAEVDG <u>T</u> C(Y) <sup>b</sup>	2183.0714 (2346.1348) <sup>b</sup>
Human IgA2	N166	N47	VFPLSLDSTPQDNVV <u>V</u> ACL <u>V</u> Q GFFPEPLSVT <u>W</u> SESGQ <u>N</u> VTAR	4533.252616
Human IgA2	N211	N92 <sup>c</sup>	HYTN <u>S</u> SQDVT <u>V</u> PCR <sup>c</sup>	1605.720554 <sup>c</sup>
Human IgA2	N263	N131	LSLHRPALEDLLGSEAN <u>L</u> TCTLTGLR	2962.5909
Human IgA2	N337	N205	TPLTAN <u>I</u> T <u>K</u>	957.5495
Human IgA2	N459	N327 <sup>b</sup>	LAGKP <u>T</u> HV <u>N</u> VS <u>V</u> MAEVDG <u>T</u> C(Y) <sup>b,d</sup>	2183.0714 <sup>d</sup> (2346.1348) <sup>b</sup>
			MAGKP <u>T</u> H <u>I</u> N <u>V</u> S <u>V</u> MAEADG <u>T</u> C(Y) <sup>b,d</sup>	2129.9908 <sup>d</sup> (2293.0541) <sup>b</sup>

<sup>a</sup>Depending on the IgG3 allotype (Vidarsson et al. 2014).

<sup>b</sup>This glycosylation site is naturally found on a peptide with and without the C-terminal tyrosine (Klapoetke et al. 2011; Bondt et al. 2016).

<sup>c</sup>This glycosylation site is only present on the IgA2 allotype A2m(1) (Tsuzukida et al. 1979).

<sup>d</sup>Naturally occurring polymorphisms are known for this IgA2 peptide portion: I326→V and A335→V (Torano and Putnam 1978; Tsuzukida et al. 1979; Deshpande et al. 2010).

Both MS- and tandem MS-based glycopeptide methods are used for the (relative) quantitative profiling of immunoglobulin glycosylation. When no fragmentation is performed, glycopeptides are identified based on accurate mass and isotopic pattern, and targeted data extraction can be performed with dedicated software tools, such as LacyTools (Jansen et al. 2016). Alternatively, targeted tandem MS methods, like selected reaction monitoring (SRM) or product-ion analysis (PIA), also sometimes called parallel reaction monitoring, can be used. In both modes, strongly linked to unit mass resolution (quadrupole or ion trap) or high resolution (time-of-flight, orbitrap or ion cyclotron resonance) mass analyzers, respectively, only the ion of interest (precursor) is transferred to the collision cell. A specific transition is then monitored by selecting a product ion either in the second mass analyzer (SRM) or during data processing (PIA). Usually, an intense oxonium ion is selected during SRM, while the inclusion of additional, more specific transitions such as glycopeptide Y-ions is advisable in order to secure the specificity of these targeted approaches. Targeted tandem MS methods are well suited for complex samples and were recently described for the relative quantification of IgG, IgA and IgM glycopeptides from plasma, without the requirement of an immunoglobulin enrichment step (Hong et al. 2015; Yuan et al. 2015). The application of such a method on a clinical cohort of epithelial ovarian cancer patients showed the differential expression of glycopeptides from all of these antibodies (Ruhaak et al. 2016). For both targeted and untargeted MS methods, the relative quantification of glycoforms is dependent on some form of normalization. Most often, total area normalization is used, which

has as a downside that the abundancies of individual glycoforms observed are not independent, i.e. when the absolute abundance of one glycoform increases, other glycoforms will decrease in relative abundance although their absolute abundance is unchanged. Additionally, while differences in ionization and detection efficiency of glycopeptides with the same peptide backbone can be minimized in MS, intrinsic differences in their fragmentation result in large biases in relative tandem MS analysis and make total area normalization ill-suited for tandem MS approaches. The independent quantification of glycoforms can be obtained by using heavy isotope labeled internal standards. Such standards have the additional potential to improve method robustness. While an ideal internal standard would cover the complete repertoire of different glycoforms per immunoglobulin glycosylation site, obtaining these standards is still challenging. Recent attempts focused on the use of heavy-labeled IgG glycopeptides containing only one GlcNAc for the quantification of IgG subclasses and glycoforms (Roy et al. 2018). Alternatively, glycopeptide functional groups were employed for the introduction of an external heavy (for the internal standard) or light (for the studied samples) label to the analytes, using, for example, heavy-labeled variants of the amine reactive benzoic acid *N*-hydroxysuccinimide, succinic anhydride or TMT (Ye et al. 2013; Kuroguchi and Amano 2014; Pabst et al. 2016). The latter was never described for its use with immunoglobulin glycopeptides, but provides the opportunity to multiplex up to 10 samples by labeling them with different isobaric tags which allows their quantification after MS fragmentation (Ye et al. 2013). Another strategy was recently used for the comparison between monoclonal



antibody glycosylation of biosimilars and their innovator product by modifying the C-terminus of glycopeptides with  $^{18}\text{O}$  during the tryptic digestion of the protein in  $\text{H}_2^{18}\text{O}$  (Srikanth et al. 2017). Finally, the development of intact heavy-labeled antibodies might be a suitable source of heavy-labeled glycopeptides in the future, especially when their glycosylation resembles human antibody glycosylation. Intact protein standards have the advantage over glycopeptide standards that they can be introduced in the sample preparation protocol at an early stage, enabling correction for additional sample processing steps.

### Intact glycoproteins and large protein fragments

The analysis of intact glycoproteins by MS is a powerful method for the characterization of proteoforms, including the combination of different glycosylation sites and other post-translational modifications (PTMs). Although this is a substantial advantage over the methods described above, the large glycan heterogeneity in combination with the numerous charge states in which the intact glycoproteins occur in ESI-MS make in-depth analysis challenging. Additionally, efficient tandem MS of intact antibodies is still in its infancy and the resolving power of commonly used separation methods is less developed for glycoproteins as compared to glycopeptides. This means that, similar to released glycan analysis, site-specificity is often lost. Luckily, new developments addressing these challenges have recently accelerated the intact and top-down analysis of monoclonal IgGs (Periat et al. 2016; Toby et al. 2016; Bobaly et al. 2018; Goyon, Francois, et al. 2018; van der Burgt et al. 2019). Additionally, so-called middle-up and middle-down approaches, in which the Fab portion is cleaved from the Fc portion prior to MS analysis, provide another solution for the more in-depth characterization of IgG and its PTMs (Resemann et al. 2016).

The major technical developments in the field of intact antibody characterization involved new and improved high resolution MS methods (Toby et al. 2016). MS approaches generally used include Orbitrap-MS (Rosati et al. 2012), Fourier transform ion cyclotron resonance (FTICR)-MS (Nicolardi et al. 2014) and high resolution time-of-flight (TOF)-MS (Haselberg et al. 2018). Tandem MS of intact glycoproteins is complicated by the fact that fragmentation strategies which are commonly used for glycopeptides, like CID, ETD and ECD (or combinations thereof), often result in inefficient fragmentation. However, using Orbitrap-MS in combination with ETD and EThcD (a combination of electron transfer dissociation and higher energy collisional dissociation) after ESI, fragmentation was achieved of therapeutic IgG1 and IgG2 molecules, showing a sequence coverage of approximately 30% (Fornelli et al. 2017). Alternatively, using MALDI-in-source decay-FTICR-MS for a NIST monoclonal antibody reference material, 31% and 65% sequence coverage was achieved for the heavy and the light chain, respectively (van der Burgt et al. 2019).

Intact antibody samples as well as antibody-based biopharmaceuticals can be directly analyzed by MALDI-MS or via direct infusion with ESI-MS (Yang et al. 2017; Wohlschlagel et al. 2018; van der Burgt et al. 2019). However, proteoforms are often (partly) separated prior to ESI-MS by LC or CE to reduce the complexity of the MS data. Various LC modes hyphenated to MS are reported for the intact or top-down characterization of mAbs, including RP-LC and HILIC (Periat et al. 2016; Bobaly et al. 2018). While RP-LC provides efficient separation of mAbs based on their hydrophobicity and is often used to assess biopharmaceutical protein degradation or misfolding (Rathore 2009), HILIC is well suited for the analysis

of glycoproteins as it allows glycoform separation and provides a high sensitivity with MS (Periat et al. 2016). Alternative LC methods for glycoprotein separation include size exclusion chromatography (SEC), ion-exchange chromatography and hydrophobic interaction chromatography (Fekete et al. 2017). Additionally, these can be used as first dimension in 2D-LC-MS (Fekete et al. 2017). For example, SEC is very powerful for the characterization of protein aggregation and may precede RP-LC for MS analysis (Goyon, Sciascera, et al. 2018). An exciting recent development is the coupling of affinity chromatography to MS, providing information on the interaction of specific proteoforms of IgG with, for example, the Fc neonatal receptor (Gahoual et al. 2017). Besides LC, CE is also highly suitable for intact mAb separation, providing information on antibody charge variants including deamidated products (Goyon, Francois, et al. 2018; Haselberg et al. 2018).

Sample preparation for the intact analysis of IgGs often involves the purification and desalting of the antibody prior to injection into the system. Alternatively, to reduce complexity, specific enzymes may be used that cleave in between the Fab and Fc portion, or disulfide bridges of the antibody may simply be reduced, yielding the two light and the two heavy chains separately. The MS profiling of the resulting fragments is referred to as middle-up, while the subsequent MS fragmentation of the protein fragments, to obtain sequence information, is called middle-down (Resemann et al. 2016). Examples of enzymes used in these approaches are IdeS, which is expected to result in two Fc/2 fragments and one intact F(ab')<sub>2</sub> fragment by cleaving IgG under the hinge region, and GingisKHAN which results in one intact Fc and two intact Fab fragments by cleaving IgG above the hinge region (van der Burgt et al. 2019). Both MALDI-MS and direct infusion ESI-MS perform quite well for the profiling of IgG Fc glycoforms, providing similar results to released glycan and glycopeptide methods, albeit with less analytical depth in terms of number of glycoforms covered (Reusch, Habegger, Falck, et al. 2015; van der Burgt et al. 2019). Besides obtaining higher resolution both in separations as well as mass analysis (Stoll et al. 2018), the cleavage of Fc and Fab is especially useful for Fab glycosylated antibodies. For example, for the marketed therapeutic mAb Cetuximab a significant reduction in data complexity was reported after treatment with IdeS (Dai and Zhang 2018). Additionally, it enables the characterization of polyclonal Fc glycosylation by removing the hyper variable Fab portion (Leblanc et al. 2014). Finally, the middle-up characterization of IdeS digested antibodies by HILIC-ESI-MS proved to be suitable for monitoring drug-antibody coupling for antibody-drug conjugates (D'Atri et al. 2018). Approaches similar to the ones described above for IgG may be suitable for the middle-up or middle-down characterization of IgA1 and IgD glycosylation using the O-glycan proteases reported to cleave N-terminal of O-glycosylation sites (Yang, Ao, et al. 2018). Such an enzyme would be able to separate Fc from Fab of these antibodies by cleaving in the hinge region.

### Other approaches

Traditionally, glycans, or better glycan motifs, have been analyzed with lectins or anti-carbohydrate antibodies (Table I). Due to the low specificity, especially of lectins, this has often made conclusions difficult or has even led to misinterpretations (Hendrickson and Zherdev 2018). Nonetheless, the simplicity and low implementation hurdle make the approach attractive, especially for routine applications, if drawbacks can be managed. For example, lectin microarrays can achieve increased specificity by relying on multiple binding hits to lectins with overlapping specificities (Cook et al. 2015). Additionally,

they can be used in fingerprinting approaches focusing on emerging differences, rendering specificity less important, as demonstrated for the differentiation of therapeutic mAb critical quality attributes (Zhang et al. 2016). Increasing the specificity of individual affinity probes by chemical biology is another approach which employs, for example, engineered lectins and catalytically silent glycosidases (Arnaud et al. 2013; Lectenz<sup>®</sup>Bio 2019).

Glycoengineering has opened up many new possibilities to study structure–function relationships of antibody glycosylation. In recent years, several groups have engaged in extensive glycoform-resolved functional studies using biochemical instead of molecular detection. Surface plasmon resonance (SPR) and affinity chromatography with UV detection have been used to unravel the complex influence of IgG Fc glycans on receptor binding, including Fc gamma receptor (Fc $\gamma$ R), fetal/neonatal Fc receptor and complement factors (Dashivets et al. 2015; Thomann et al. 2015; Subedi and Barb 2016; Dekkers et al. 2017; Wada et al. 2019; Table I). This was often complemented with biological assays for antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, confirming the glycan impact on these important immunological responses.

A strong three-dimensional structural basis for these studies has been provided through the crystallographic analysis of IgG alone and in complex with Fc $\gamma$ R. This remarkably showed glycan–glycan and glycan–protein interactions within IgG and between IgG and the Fc $\gamma$ R (Krapp et al. 2003; Ferrara et al. 2011). In recent years, this basis has been refined by protein nuclear magnetic resonance spectroscopy (NMR) studies (Table I). With a consequential focus on solution phase structures and more flexible protein regions, the impact of the IgG Fc glycan on the organization of the N<sub>297</sub>-containing C'E loop has been highlighted as a critical factor for Fc $\gamma$ R binding (Subedi and Barb 2015). Additionally, the structural basis for the outstanding role of IgG Fc core fucosylation has been refined (Falconer et al. 2018). Based on the detailed findings of the IgG glycan functional impact and indications of the Fc $\gamma$ R glycan importance in its interaction with IgG (Hayes et al. 2014), natural human Fc $\gamma$ R glycosylation and the functional impact of Fc $\gamma$ R glycoforms are becoming a focus of attention (Hayes et al. 2017; Patel et al. 2018; Yagi et al. 2018; Washburn et al. 2019).

## Perspectives

Most research on antibody glycosylation is performed for endogenous IgG derived from plasma or recombinant IgG from culture broths in the biopharma industry. IgG is the most abundant antibody isotype in the human circulation and a lot is known about its role in the humoral immune system. Furthermore, there are many IgG-based biopharmaceuticals and the number continues to grow. However, this does not mean that the glycosylation analysis of other immunoglobulin isotypes, or of antibodies derived from other body fluids, is less important. That still little is known on the behavior and effect of the glycosylation of antibodies other than IgG in health or disease is likely a consequence of their lower abundance in the circulation and the higher complexity of their glycosylation. Furthermore, sometimes it may be more relevant to study antibodies in biofluids other than plasma, such as saliva, synovial fluid or cerebrospinal fluid, or even in tissue. However, this is hampered by low concentrations or poor accessibility of these samples.

Recent developments in MS-based technologies, like the high sensitivity nano-LC-ESI-MS and CE-ESI-MS analysis of glycopeptides, provide the platforms to bring the field of global antibody glyco-proteomics further. Steps should be taken for the miniaturization

of the sample preparation, most importantly for low abundance antibodies like IgD and IgE, of which the latter specifically is gaining more and more interest in the field of allergy research (Shade et al. 2019). Furthermore, miniaturization of the sample preparation is of value when antigen-specific sub-populations of antibodies are studied (Kapur et al. 2014) as well as for antibodies derived from small-scale clonal in vitro cultures. The latter may provide valuable insights into the regulation of immunoglobulin microheterogeneity.

Similar to IgG, IgA glycosylation can currently be routinely profiled at the glycopeptide level in biomedical settings (Plomp et al. 2018; Chandler et al. 2019). Applications of this method may be particularly relevant for diseases with mucosal involvement, such as inflammatory bowel diseases and colon cancer, where the glycosylation of secretory IgA can be compared to its blood counterpart.

While glycopeptide analysis is key in the site-specific characterization of immunoglobulin glycosylation, challenges remain with respect to the full structural characterization of all glycoforms as well as comprehensive proteoform analysis. The prior can be addressed by combining a glycoproteomic approach with a glycomic approach, releasing the glycans from purified immunoglobulins and subjecting them to powerful isomer separation techniques like PGC-LC or CE prior to tandem MS. The current developments in IMS may very well lead to its integration in such characterization workflows, both on the glycan and glycopeptide level. Especially, the smart integration of IMS with liquid phase separations, mass spectrometry and fragmentation techniques shows great promises of orthogonality for structural analysis. Comprehensive proteoform analysis can be addressed by MS-based intact and middle-up approaches. On the level of glycosylation, we expect a method that allows the analysis of an intact Fc portion, for example via IgG digestion by GingisKHAN, to provide valuable information on the combinations of glycoforms present on the heavy chain Fc dimer. Additionally, information will be obtained regarding other PTMs of the antibody, such as oxidation, deamidation, glycation and proteolytic truncation that may influence immunoglobulin effector functions and half-life. However, classical peptide mapping, which is modified to accurately include PTMs, will be essential to warrant the site-specific analysis of co-occurring PTMs (Choi et al. 2017). While this may also be efficiently addressed by top-down or middle-down approaches in the far future, these are unlikely to provide a sole solution for all relevant PTMs.

To advance the in-depth and high-throughput screening of high numbers of samples in biomedical research, progress should be made in the robustness and automation of current methods. Additionally, as antibody glycosylation is expected to play a role in future clinical diagnostics, efforts should be made to simplify current workflows and adjust them to clinical diagnostic platforms that are currently in use. Here, one can think about simplification of sampling methods, for example by using dried blood spots (Gudelj et al. 2015; Choi et al. 2017). Furthermore, specifically for IgG Fc galactosylation, which is a promising candidate for the monitoring of low grade inflammation, the prediction of disease prognosis, and for treatment monitoring in various conditions (Kemna et al. 2017; Plomp et al. 2017; Simurina et al. 2018), a CE-LIF-based method was recently developed for the analysis of EndoS released glycans (Vanderschaeghe et al. 2018). This method does not require the enrichment of the antibody prior to glycan release and provides an example of a suitable tool for the routine profiling of IgG Fc galactosylation in a clinical setting. With regard to MS-based methods, important steps would be the implementation of isotope labeled internal standards early in the sample preparation process and the simplification of sample preparation protocols. Sample throughput and information density

may be gained by the integrated analysis of different immunoglobulin classes. This was shown to be possible via the direct digestion of the complete plasma proteome, in combination with a targeted MS approach for IgG, IgA and IgM glycopeptides (Hong et al. 2015). Alternatively, we expect a more in-depth characterization of immunoglobulin glycosylation microheterogeneity when various antibodies are simultaneously enriched from the same sample prior to glycopeptide generation.

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## Conflict of interest statement.

The authors declare no conflict of interest.

## Abbreviations

2-AB, 2-aminobenzamide; AC-UV, affinity chromatography with UV detection; AEX, anion exchange; APTS, aminopyrene trisulfonate; CCS, collision cross section; CGE-LIF, capillary gel electrophoresis with laser-induced FLU; CID, collision-induced dissociation; CZE, capillary zone electrophoresis; ECD, electron-capture dissociation; ESI, electrospray ionization; ETD, electron-transfer dissociation; FLU, fluorescence; FTICR, Fourier transform ion cyclotron resonance; HILIC, hydrophilic interaction liquid chromatography; IMS, ion mobility spectrometry; mAbs, monoclonal antibodies; MS, mass spectrometry; PGC, porous graphitized carbon; PIA, production analysis; RP, reversed phase; SEC, size exclusion chromatography; SPR, Surface plasmon resonance; SRM, selected reaction monitoring; TMT, tandem mass tag.

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