Antibody glycomics signatures of SARS-CoV-2 infection and vaccination

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DISCUSSION

SARS-CoV-2, the virus that is affecting our lives through the global pandemic, enters the human body by attaching to its receptor, angiotensin-converting enzyme 2, on airway cells via its so-called spike protein. As the spike protein sticks out from viral cell surfaces in high abundance, it is one of the major antigenic determinant a potent antibody response is elicited against. Whilst most infected individuals clear the virus and recover rapidly, a number of patients develop life-threatening conditions, that seem to coincide with the awakening of the adaptive immune response and subsequent ADCC via FcγRIII receptors. What factors steer inter-individual differences in the disease course remains to be further investigated, but an important driver may be antibody glycosylation[1]. Changes in the glycosylation of pathogen-specific antibodies have been described both in natural infections and vaccinations, highlighting their biomarker potential[1-4]. In this thesis, antibody glycomics signatures were explored in SARS-CoV-2 infection and vaccination using LC hyphenated to MS. The used platform has been highly attractive for studying IgG glycosylation, as it readily provides subclass- and site-specific information. Using this method, the screening strategy and early severity marker potential of anti-S IgG1 glycosylation for early risk stratification of SARS-CoV-2 patients has been evaluated, resulting in the identification of high bisection and low galactosylation and sialylation as major predictors for ICU admission at time of hospitalization, and low fucosylation as a general glyco-phenotype characterizing hospitalized patients **(Chapter 2)**. Early detection of a pro-inflammatory glycosylation pattern may provide a broader intervention window and decreased number of ICU-admissions. Furthermore, the glycosylation of antibodies upon mRNA vaccination of antigen-naïve and antigen-experienced individuals has been explored, where a transient afucosylated IgG response to the BNT162b2 mRNA vaccine was observed in the former but not in the latter group, which was reflected in FUT8 expression and as well predicted antibody titers upon the second dose **(Chapter 3)**. The severity and efficacy marker potential of glycosylation signatures such as fucosylation, bisection and galactosylation, suggest the need for the development of more widely available platforms to study pathogen-specific IgG glycosylation as a biomarker and as well stimulate the study of mechanisms underlying the regulation of glycosylation in B cells in COVID-19 and beyond.

7.1 IgG glycosylation analysis beyond MS

The current method used for the assessment of IgG glycosylation is considered as a bottom-up glycomics approach, using LC-MS to relatively quantify tryptic IgG glycopeptides. This platform has been pivotal to study SARS-CoV-2 S protein-specific IgG glycosylation **(Chapter 2)**, although it requires a special infrastructure that is reserved to dedicated laboratories.

Similarly to this thesis, numerous initial glycan biomarkers are proposed based on MS-based studies, but these findings are rarely propelled from the discovery stage to clinical implementation[5,6]. Hence, the question arises whether alternative orthogonal techniques replacing such a high-end analytical platform are available, that may solely focus on established glycan biomarkers of COVID, but with customization potential to other pathogens and antigens – and are easier to validate and implement into a clinical environment than MS. The desired technique would ideally require low number of sample preparation steps, and be instrumentally simple, quantitative, relatively fast, cost-efficient and suitable for automation and high-throughput workflows. At the same time, such techniques should retain IgG subclass- and site-specificity, and be specific for a subset of glycoforms of interest, for example those with and without a core-fucose or bisected GlcNAc.

7.2 Enzyme-linked immunosorbent assays

Appealing and straightforward alternatives to LC-MS are antibody-based approaches such as enzyme-linked immunosorbent assays (ELISAs). Immunoassays have long been considered as standard methods for biomarker quantification and largely match the above described requirements, but have hitherto not been utilized in glycomics. A recently developed two-tier ELISA method, termed fucose-sensitive ELISA for antigen-specific IgG (FEASI), shows some similarities with the approach used for affinity-capturing anti-S IgG in this thesis (**Chapter 2** and **3**), with demonstrated comparability to the used LC-MS method. This setup allowed to simultaneously determine quantitative and qualitative anti-S IgG responses[Sustic et. al. under revision], and thereby opens new avenues for the rapid assessment of fucosylation levels in virtually any immune response thanks to its straightforward customization potential to other antigen specificities. The fucose-sensitive Fc interaction partner FcγRIIIa used in the FEASI assay is inherently site-specific, and likewise specific to the IgG1 and IgG3 subclasses, as FcγRIIIa hardly binds IgG2 and IgG4[7]. However, a general drawback of immunoassays is their proneness to produce incorrect results due to too low, or too high levels (Hook effect) of antigens or antibodies, compromising sensitivity and specificity.

7.3 Lectin-based assays

Another clinically accessible alternative with similarly attractive properties to ELISA are lectin-based immunoassays. Lectins are glycan-binding proteins that recognize and reversibly bind to specific glycan motifs[8]. Historically, lectins (also known as agglutinins) were found to

distinguish human ABO blood groups by Karl Landsteiner[9]. Unknown at the time, the serologic differentiators between blood groups still important in blood transfusions, were *O*-glycans. Besides, lectins have traditionally been used for glycan/glycoprotein enrichment purposes[8]. Furthermore, there has been a widespread use of lectin-based assays for the analysis protein glycosylation, including that of IgG[10]. Recently, anti-S and anti-N IgG fucosylation of COVID-19 patients have been investigated after their affinity-enrichment using a lectin-affinity assay. This assay exploited the lectin *Aleuria aurantia,* specific for fucose residues linked α1,6 to *N*-acetylglucosamine. The comparability of this assay to LC-MS awaits confirmation. Different lectins exhibit high affinity binding to different glycan determinants, varying in specificity from single monosaccharides to glycans with more complex structural features[8]. Hence, one could consider their use highly promising for IgG glycosylation analysis. However, the concern about lectin-based methods lies in their inherent lack of site-specificity. For example, the Fab portion of antibodies often carries diantennary glycans with high sialylation, and it has been suggested that these may even be better reachable by the lectin *Sambucus nigra agglutinin*[11]. This lectin has been used to assess both total and donor-specific IgG sialylation rates in a transplantation setting[12,13], and likely led to a quantification bias. Similar concerns apply for the use of any lectin for IgG glycosylation analysis, including *Aleuria aurantia* for the assessment of anti-S IgG fucosylation.

7.4 Glycosidase-plate based assays

Glycosidase plate-based assays may also be good candidates to substitute high-end analytical platforms. For example, Rebello et al. introduced a novel chemo-enzymatic assay for the absolute quantification of galactosylation and sialylation[14]. In this study, affinity-purified IgG has been exposed to parallel galactosidase and sialidase treatments. During a subsequent reaction, a fluorescent compound was formed, of which readout was quantitative for the amount of terminal and total galactosylation and, in-turn, sialylation of IgG. Using samples of patients with inflammatory bowel disease, a condition in which low IgG galactosylation has been observed[15], the authors additionally demonstrated that the low galactosylation index obtained with the glycosidase plate-based assay was highly comparable to that obtained by HILIC-FLD-MS[14], a well-established technique to assess *N*-glycosylation on the released glycan level[16]. While this approach has been developed to determine sialylation and galactosylation levels, its implementation to other glycosylation traits has not yet been established.

7.5 The bottleneck and suggestions for future development

A common drawback of all the afore described assays is that in their current form they cannot fully reveal differences between the four IgG subclasses, of which in general, differences have been shown on the level of both total and antigen-specific IgG[4,17,18]. Therefore, a concentration-dependent change in one subclass may mask alterations of glycosylation specific to another subclass. As the nature of the antigen may induce qualitative and/or quantitative changes only in a single subclass, this potential bias could lead to both false interpretations and undetected changes relevant from a glyco-immunological aspect. One way to overcome the limitation of subclass ambiguity could be the addition of a subclass-specific affinity purification step. Antibodies specific to human IgG subclasses are commercially available, and for example have been used to determine SARS-CoV-2 anti-RBD IgG subclass titers in an ELISA setup[19], although evaluation of glycosylation for all four subclasses would upscale experiments, costs and sample amount requirement. Hence, following this direction it may be better to affinity-enrich a pre-selected subclass for analysis based on prior knowledge on the nature and/or context of the antigen the antibodies are specific for, from which assumptions can be made on expected subclass-specific changes in glycosylation. For example, protein antigens largely induce an IgG1 and IgG3 response, and when embedded into plasma membranes, such as the S protein is, an afucosylated response may occur, as has been shown in alloimmune and infectious diseases[4]. On the other hand, carbohydrate antigens or allergens, present in a soluble form, mostly result in IgG2 and IgG4, respectively, with negligible amount of afucosylation[17,20]. Moreover, antibody responses in the early stages of SARS-CoV-2 infection are known to mainly result in the IgG1 subclass[21]. To date, however, there are no non-MS protocols describing IgG Fc glycosylation analysis preceded by affinity-enrichment of a single subclass, which is encouraged in future studies aiming for the development of such assays with the intention of clinical implementation.

An additional shortcoming which lectin- and glycosidase plate-based approaches share with separation- and MS-based released glycan analysis is their lack of site specificity. Besides the conserved Fc glycosylation site of IgG, ~15-25% of circulatory IgG has been described to be glycosylated in the variable domain of the Fab[22]. Treatment of immobilized antibodies with IdeS (FabRICATOR®)[23] or IdeZ[24] could serve as a potential solution to distinguish between Fc and Fab glycans, as digestion with these IgG-specific enzymes leads to cleavage below the hinge region, releasing the Fc. This latter approach has been successfully used to study released glycans of anti-S IgG originating from mother-baby dyads by CGE-LIF[25]. However, it is

important to note that using this approach, glycans originating from glycosylation sites other than N297 in the Fc moiety of IgG3[20,26,27] may contribute to the observed glycosylation pattern.

As compared to MS, these assays are ill-suited to serve as powerful multi-analyte single assays so advantageous in early biomarker discovery **(Chapter 2)**, but as they focus on a single determinant, are better suited as simplistic assays for clinical application after their further development and validation. While there has clearly been progress toward the democratization of glycosylation analysis using such non-MS-based approaches, none of them are capable of providing an unambiguous, comprehensive overview on glycosylation features of IgG in a subclass- and site-specific manner in their present state. Hence, such simplistic techniques for the assessment of (antigen-specific) IgG glycosylation currently hold promise as initial screening assays rather than readily available diagnostic tools.

7.6 The suitability of MS for clinical application and biomarker discovery

Noteworthy exceptions exist, but MS-based technologies have hitherto been underutilized in clinical practice[28]. Examples of FDA-approved clinical applications are the Biotyper, which is a MALDI-TOF-MS device for microbial identification from human cultures[29], and two HPLC-MS/MS techniques: 1) for screening of inborn metabolic disorders as part of newborn screening programs, and 2) for monitoring concentrations of the therapeutic drug tacrolimus, respectively. All of these technologies make use of some sort of benchmark: mass spectra obtained with the Biotyper is compared to a spectral library from known microorganisms. For new-born screening, isotopically labelled internal standards are included at the preanalytical stage. Tacrolimus quantitation is performed by establishing a calibration curve with known amounts of tacrolimus spiked into freeze-dried whole blood. Another FDA-approved clinical assay is becoming the gold standard of vitamin D quantification[30]. Other, non-FDA-approved clinical applications likewise relying on LC-MS/MS encompass therapeutic drug monitoring assays including those for monoclonal antibodies (e.g. Infliximab), but also steroid hormones and protein markers such as immunoglobulin G subclasses, thyroglobulin, or glycosylation of transferrin for the detection of congenital disorders of glycosylation[31,32]. From these examples it is obvious that LC-MS/MS excels at small molecule, peptide and protein marker identification. However, during the entry of these systems to the clinic, specific regulatory challenges were faced with regards to analytical validation, quality control materials and standards, intended use and robustness of software and data analysis tools[28]. It is beyond the scope of this thesis to discuss all these aspects, as are thoroughly elaborated elsewhere[6], it is of

note that failing to fulfil any of the above criteria can vastly hamper entry of MS-based techniques into the clinic. The clinical implementation of the above examples were the result of extensive communication of test developers and instrument manufacturers, and particularly for FDA-approved assays, with the regulatory authorities from the early stages of development[28,32]. As the reader may have noticed, none of these few (FDA-approved) MS-techniques measure glycoproteins or fragments thereof. Unsurprisingly, as given the immense heterogeneity and subsequent substochiometric nature of these types of biomolecules, isotopically labelled standards (for the entire glycoform repertoire) and quality control materials – both of which are major requirements for clinical implementation of MS – are scarcely available, limiting their widespread application in diagnostics laboratories[6,28].

Efforts may be made to transfer the here introduced, MS-based IgG glycopeptide analysis method to the diagnostic laboratory in its current, or alternatively, in an instrumentally more simplistic form. However, as mentioned, the afore described limitations and requirements involved toward clinical implementation hold most MS-based techniques measuring glycan biomarkers at the biomarker discovery stage. By contrast to the limitations of the assay-based methods described in the previous section, bottom-up glycomics workflows, such as the implemented LC-MS-based method, can be readily and reliably used for site- and subclass-specific analysis of IgG glycosylation in a single measurement **(Chapter 2)**. Similar methods have been developed to fathom the proteome and glycoproteome for biomarkers in various discovery studies[33-35], exemplifying the advantages and essentiality of MS in a high-throughput biomarker discovery setting, rather than in diagnostic assay development for clinical applications.

7.7 Mass spectrometry-based IgG glycopeptide analysis

7.7.1 LC-MS

As described in **Chapter 2**, the reversed-phase LC-MS-based workflow implemented for the analysis of anti-S IgG glycosylation has been used in a broad range of (disease) settings before. The workflow is relatively robust, as can be used to analyze IgG from various sources and body fluids[17,36-39], and has also been optimized for the analysis of IgA[40]. Following the affinity-enrichment of total and/or antigen-specific IgG, the applied workflow continues with tryptic digestion, producing the peptide backbones EEQ*Y***N**ST*Y*R, EEQ*F***N**ST*Y*R and EEQ*F***N**ST*F*R for IgG1, 4, and 2/3, respectively. Using reversed-phase LC-MS with prior sample pre-

fractionation on an on-line solid-phase extraction column, the obtained IgG glycopeptides – stripped from salts and contaminants – elute according to their hydrophobicity. The difference in hydrophobicity of the tryptic glycopeptides is introduced by substitutions of one or two tyrosine(s) to phenylalanine(s), resulting in the elution order as follows: IgG1, IgG4, and IgG2/3. As IgG2 and IgG3 are assumed to share the same tryptic glycopeptide in the Caucasian population[20], their profiles remain indistinguishable. Depending on the IgG3 allotype, it may also result in an overlap with IgG4, or its digestion may result in a longer glycopeptide (TKPWEEQ*Y***N**ST*F*R). Hence, in vain of prior knowledge on the IgG3 allotypes, the interpretation of glyco-patterns may be largely biased. This has been exemplified by a recent study on SARS-CoV-2-specific anti-RBD IgG glycosylation, where the IgG3 allotype has been misassigned as to belong to IgG4, leading to ill-founded conclusions[19].

7.7.2 MALDI-TOF-MS

The popularity of MALDI-TOF-MS is thanks to its instrumental simplicity, relatively low cost and amenableness to (automated) high-throughput applications[41]. Data acquisition by MALDI is fast as compared to LC-MS and it mainly generates singly charged ions[42,43], that is beneficial for simple data analysis workflows. MALDI has been popular in biomarker discovery, and interestingly, an early cancer biomarker study was also performed by MALDI[44], followed by others[45]. It has also been attractive for glycopeptide profiling[46], including those originating from IgG[47,48], resulting in remarkably comparable glyco-profiles to those obtained by HILIC-UPLC[48].

As described in **Chapters 1**, **5** and **6**, sialic acids are prone to in-source and metastable decay when analyzed with reflectron positive mode MALDI-TOF-MS. Therefore, sialylated glycoconjugates are worthwhile to be stabilized prior to their analysis. De Haan et al. overcame sialic acid fragility by developing and optimizing a one-pot one-step linkage-specific sialic acid derivatization approach for the assessment of IgG Fc glycosylation on the glycopeptide-level[48]. The innovative step in this method was the optimization of the derivatization reaction on the peptide portion. As IgG glycopeptides carry three carboxyl groups (2x E and the C-terminal), the method had to be tailor-made to avoid variable modifications and achieve uniform derivatization efficiency. While this approach was suitable to study the glycosylation of monoclonal antibodies, polyclonal IgG subclasses remained challenging to distinguish due to isomeric overlaps, stemming from the equal compositional difference (one oxygen) between phenylalanine and tyrosine, and fucose and hexoses, resulting in overlaps in the *m/z* values of

fucosylated and afucosylated IgG1 with IgG4, and IgG4 with IgG2/3. Consequently, the application of the described MALDI-TOF-MS method was impractical in a biomarker discovery setting.

To avoid subclass ambiguity, the use of a different proteases, such as chymotrypsin or GluC could be introduced to obtain larger, more specific glycopeptides. However, this would simultaneously necessitate the optimization of both the purification and derivatization protocols due to changes in hydrophilicity and raised complexity of the obtained glycopeptide backbones and reactive carboxyl groups thereon. An alternative way to avoid subclass ambiguity would be the prior fractionation by LC, or instead, the earlier suggested subclass-specific IgG affinity enrichment. This latter approach has recently been established for the subclass-specific IgG glycosylation analysis of liver fibrosis patients on the released glycan level using MALDI-FT-ICR-MS[49]. In contrast to MALDI-TOF-MS, MALDI-FT-ICR-MS instruments operate at intermediate pressure, where ions undergo collisional cooling, preventing e.g. sialic acid loss. Therefore, the modification of the method of Scott *et al.*[49] from released *N*-glycans to tryptic glycopeptides would be highly attractive for a relatively swift subclass and site-specific IgG glycosylation analysis workflow to assess severity of hospitalized COVID-19 patients based on their bisection and galactosylation signatures. Another attractive on-plate affinity-enrichment-based approach would be immuno-MALDI- (iMALDI)-TOF-MS[50] of glycopeptides, although its straightforward implementation is currently hampered by the lack of subclass-specific anti-IgG-glycopeptide antibodies.

In view of the above challenges, LC-MS remains the preferred method for clinical antibody glycomics today, as glycopeptide clusters per subclass are stretched apart over the time domain and therefore avoid isomeric overlaps, while decay caused by sialic acid loss can be minimized by the cautious selection of ion transfer voltages[51].

7.8 Mechanistic insights into linkage-specific sialic acid derivatization to support MALDI-based biomarker discovery

Various approaches have been tested to overcome the loss of sialic acids during MS analysis, such as the use of cold matrices[47], permethylation of hydroxyl groups[52] or derivatization of carboxyls[53]. The studies in **Chapter 5** and **6** built on the latter, specifically on a previously developed linkage-specific sialic acid derivatization workflow[54]. These studies were performed on glycan standards and released *N*-glycans with the aim of elucidating the reaction steps in

more detail, so that such approaches could be more rationally tailored to glycomics and glycoproteomics workflows in the future. In **Chapter 5**, the reaction space of linkage-specific sialic acid derivatization has been expanded by the introduction of catalysts formerly not tested in the field. The introduction of these novel catalysts with similar performance to, but different in physico-chemical characteristics from the conventionally used HOBt, are hypothesized to be potentially beneficial in solid-phase glycoconjugate derivatization workflows that were earlier hampered due to the incompatibility of high ethanol concentrations with e.g. PVDF membranes. While **Chapter 5** focused on a one-step linkage-specific sialic acid derivatization reaction, **Chapter 6** evaluated mechanistic aspects of two-step reactions, with particular focus on the structure and formation of lactone intermediates and their subsequent amidation. In this study, it has been shown that lactone formation is a prerequisite for the effective derivatization of α2,3-linked sialic acids which proceeds predominantly via direct aminolysis of the C2 lactone. As aminolysis is a swift reaction and requires no re-addition of a catalyst and carboxylic acid activator, these insights allow a more mindful experimental design and potentiate significantly shorter reaction times.

An interesting setting that awaits its transfer to MALDI-based biomarker discovery studies is intact glycoprotein derivatization. In a previous report, solid-phase linkage-specific sialic acid derivatization protocol has been shown to lead to efficient modification of various serum-derived intact glycoproteins, allowing their sialic acid linkage isomer differentiation whilst retaining site-specificity[55]. Performing the reaction on the intact protein level may overcome some of the limitations of glycopeptide-based liquid-phase protocols, such as variable modifications induced by a flanking C-terminus[48], and will potentially pave the way toward high-throughput glycomics applications.

7.9 Mechanistic insights into the regulation of IgG glycosylation in vaccination

As described in **Chapter 2**, various heritable and non-heritable factors cause considerable inter-individual heterogeneity in the IgG glyco-repertoire. Differential IgG glycosylation is suggested to determine inflammatory thresholds and thereby mark the inflammatory state and/or the susceptibility to inflammatory diseases of an individual[3,56-58]. Particular shifts in pathogen-specific IgG Fc glycosylation have been observed in infectious diseases and vaccinations thereagainst, suggesting the role of differential glycosylation in the regulation of the immune response[3]. What factors govern IgG glycosylation *in vivo* in B cells remains one of

the outstanding questions in the field, and vaccination provides an excellent setting to study this **(Chapter 3)**.

In **Chapter 3** we found that mRNA vaccination, resulting in spike protein presentation on host cell membranes not only induces transient afucosylated IgG responses in naïve vaccinees upon the first dose similarly to a natural infection, but re-induces afucosylated IgG generation in antigen-experienced individuals. The availability of glycosyltransferases has been suggested as one of the determinant of glycoform heterogeneity[59], and indeed, afucosylated responses in naïve individuals correlated with low FUT8 expression, restricted to a $CD27^{low}$ $CD138^{-}$ IgG^{+} $CD38^{+}$ PC antigen-specific B cell population. While this finding is clearly strengthens the observation on the effect of the antigen-context in the induction of afucosylated IgG responses[4], what remains unknown is the underlying mechanism leading to low FUT8 expression. One of the few genetic studies performed to explore the regulation of glycosylation has shown that the expression of fucosyltransferases is under the control of Hepatocyte Nuclear Factor 1α (HNF1α), of which presence upregulates guanosine 5'-diphosphofucose (GDP-fucose) synthesis[60], although epigenetic silencing of the HNF1α gene did not lead to significant associations with fucosylation of total IgG[61], which is potentially explained by the dominant presence of this transcription factor in the liver. Another study suggested that FUT8 can be also regulated via microRNA-mediated mechanisms[62]. It would be highly interesting to study if the antigen context influences microRNA transcription patterns that may suppress FUT8 expression in SARS-CoV-2 S protein-specific B cell populations and beyond.

Another interesting question regards the origin of glycan microheterogeneity on pathogen-specific IgG. In **Chapter 3**, we reported that high ST6GAL1 (α2,6-sialyltransferase) expression was characteristic to $CD27^{+}$ $CD138^{+}$ and the lowest in $CD27^{low}$ $CD138^{-}$ IgG^{+} PC subset (the one with low FUT8 expression), which has been reflected by anti-S glycosylation patterns by MS **(Chapter 3)**, suggesting that different B cell populations generate the heterogeneity in the glycosylation of IgG.

Lastly, with the growing interest in Fc-mediated effector functions – such as ADCC, antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent complement deposition (ADCD) due to their role in the control and clearance of infectious agents[63-65], identification of pathogen-specific antibody glycosylation signatures that may prognose their ability to evoke effector functions would be a highly interesting research direction in the future.

7.10 References

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