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Glycomic markers of inflammatory bowel disease and IgA nephropathy

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Chapter 6

Discussion and Perspectives



Discussion and Perspectives

The work presented in this thesis has various innovative aspects. To my knowledge, the study presented in **chapter 3** is the largest analysis of the total plasma *N*-glycome (TPNG) in IBD patients, considering both the discovery and replication cohorts¹⁻³. This research is novel, including sialic acid (SA) linkage-specific information in the TPNG and in IBD. We identified multiple associations of TPNG with Crohn's disease and ulcerative colitis, largely replicated in the second cohort. Differences in glycan complexity, fucosylation, bisection, galactosylation, and sialylation were found between IBD patients and healthy controls (HCs). Novel SA linkage data showed associations with age, sex, and IBD. Furthermore, our high-throughput method revealed differences within the CD and UC groups regarding surgery, medication response, and disease localization. Established inflammation markers, such as low galactosylation in IgG and higher sialylation in acute-phase proteins, were evident in CD and UC patients. The main findings about the glycosylation changes in antennarity, galactosylation, bisection, fucosylation and sialic acids were reproduced in a recent TSNG glycan study by UPLC using fluorescent procainamide labeling on released glycans¹. That study did not include sialic acids linkage specific information like what is reported here in **chapter 3**, but they were able to report on a branching difference between CD and UC due to the different retention time observed with the α 1,3 and α 1,6-mannose arm-linked galactoses in their chromatography technique, which was not possible with our MALDI-TOF-MS approach. Looking ahead, a method that captures all these glycosylation features would be desirable, *i.e.*, resolving not only sialic acid linkage differences, but also branching differences. Combination of ion mobility with MS detection, eventually after sialic acid derivatization, may be an approach to achieve this goal. We also proposed specific proteins (e.g., IgA, haptoglobin, α 1-acid-glycoprotein) as potential targets for future glycoproteomic studies to better understand IBD pathophysiology and develop therapeutic targets.

In **chapter 5**, we applied our IgA glycopeptide analytical workflow developed for IgAN analysis (**chapter 4** to a subset of the discovery cohort of IBD patients described in **chapter 3**, and report novel associations of IgA glycosylation with IBD. Our prediction model based on IgA glycopeptides effectively distinguished CD from HCs, whilst the model showed lower discrimination power for UC versus HCs. Plasma or serum glycomic analyses, using readily available biofluids, could aid early IBD diagnosis and differentiate CD from UC, reducing the need for invasive procedures. It is interesting to see that the changes reported in the TPNG for a few glycan traits like A2FS and A2FSB were potentially dominated by changes in IgA glycosylation as indicated by the relative contribution of each major plasma protein to specific glycoforms⁴ (**Figure**

1). Indeed, these presumed associations between IgA glycosylation features and IBD were confirmed in the IBD IgA targeted study presented in **chapter 5**.

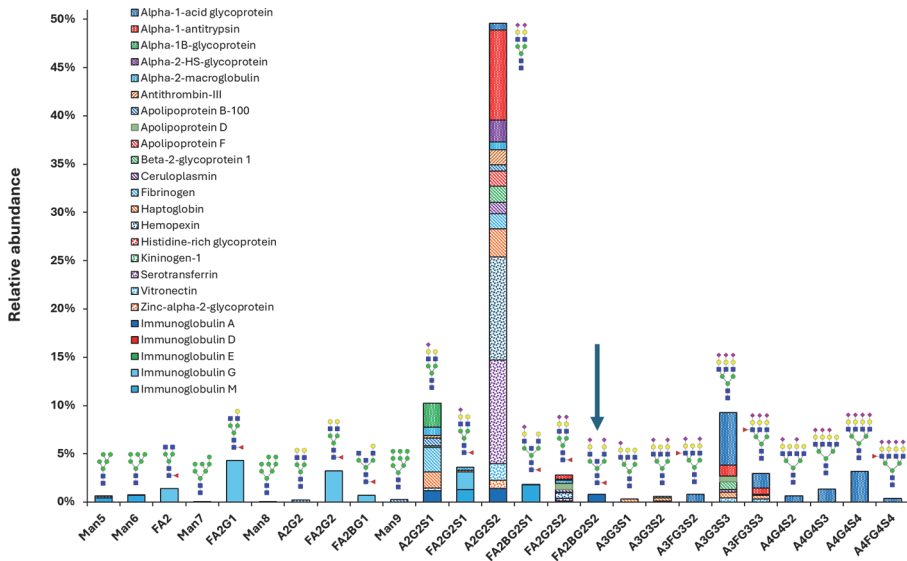


Figure 1: Estimated contribution of major blood glycoproteins to TPNG.

The contribution of IgA to FA2BG2S2 is highlighted with an arrow. The glycans contributing to FA2BG2S2 appear to be largely IgA-derived, making IgA a major contributor to the glycosylation traits A2FS and A2FSB, as postulated in the IgA specific chapters of the thesis. The figure is taken and modified from Chapter 2.

Confirming this predicted association made from findings in the non-targeted analysis of the TPNG with the targeted method on IgA shows the benefits of having a global overview of the glycosylation changes in serum, and proves the potential of TPNG analysis as starting point for biomarker research. Next to some of the IgA glycosylation changes in IBD, the well-known alterations in the high abundant IgG glycosylation with inflammation in general and specifically in IBD were also visible directly in the TPNG and confirmed in another study on the same IBD samples⁵. Unless specific proteins have already been proven to be the direct contributors or targets in specific diseases, using the TPNG analysis as starting point for investigations may be very informative.

Glycosylation's role in driving mucosal pathogenesis under chronic inflammation is an exciting and emerging research field. Current studies have shown that inflammation-induced changes in intestinal and immune cell glycosylation can produce potential prognostic biomarkers and targets for developing new therapies

to mitigate IBD-associated tissue damage. In a recent study on serum samples from IBD patients, the main glycosylation changes about antennarity, galactosylation, bisection, fucosylation and sialic acids were similarly to what is reported in **chapter 3** of this thesis¹. Their analysis goes further in the search of predictive biomarkers for disease and treatment escalation and achieved great power in case vs controls prediction as well as in escalation of treatment in high-risk vs low risk patients¹.

The analysis of the TPNG is also aligning well with genomic studies, where the effects of alterations in glycosylation coding genes affects glycosylation profiles drawing a line from coding to expression⁶⁻⁹. Overall, we observe that the insights from the TPNG presented in **chapter 2** aid in the clinical interpretation of comprehensive plasma glycosylation analyses. This review highlights the critical need for further protein-specific glycosylation studies to address the significant gaps in our current understanding and is used as reference for glycosylation background in already more than 300 research articles (scopus, July 2024).

In this thesis, the detection of rather small differences in glycosylation between CD and UC was made possible thanks to the statistical power achieved with the large number of samples used in the discovery cohort with findings being replicated in another large replication cohort (**chapter 3**). A specific challenge addressed in this work was the logistical complexity of performing sample randomization, which led to the use of a robotic platform to handle the pipetting. This included optimizing the redistribution of samples from 25 donor-specific 96-well plates, originally sorted by disease status, into new receiver plates, while maintaining balanced distributions of cases, healthy controls, technical and biological controls, and blanks. Furthermore, special care was taken to limit each donor plate to a single thaw cycle and to minimize batch effects during sample processing, ensuring to retain optimal plasma quality. Following randomization, two batches of four 96-well plates each were prepared daily and spotted onto MALDI target plates for subsequent MALDI-TOF-MS analysis.

This study shows the potential and benefits of using a robotized platform for sample randomization and sialic acid derivatization with minimum effort compared to executing this protocol by hand, including the reduction of interday batch effects which are important when handling such large numbers of samples. This workflow has already been used and adapted for similar studies focused on other diseases¹⁰⁻¹⁴. As described in **chapter 3**, it was possible to apply batch correction (performed on logarithmically transformed data) to account for the different preparation days, as well as position on row and column of the randomized samples on the 96-well plates and to remove outliers from data analysis (**Figure 2** and **Figure 3**).

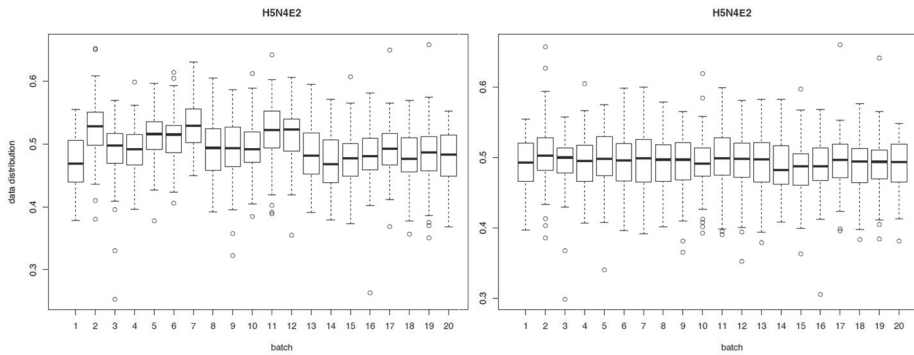


Figure 2: Batch correction applied to twenty plates of TPNG samples

Figure 2 represents the effects of batch correction (before vs. after) on all plates of the TPNG samples analyzed in **chapter 3**. This batch correction considers sample position (row, column and plate). The mean and boxplot distribution of the major TPNG glycan H5N4E2 is represented. This batch correction method aims to model and remove the variability associated with batch preparation due to technical variations while retaining biologically meaningful differences.

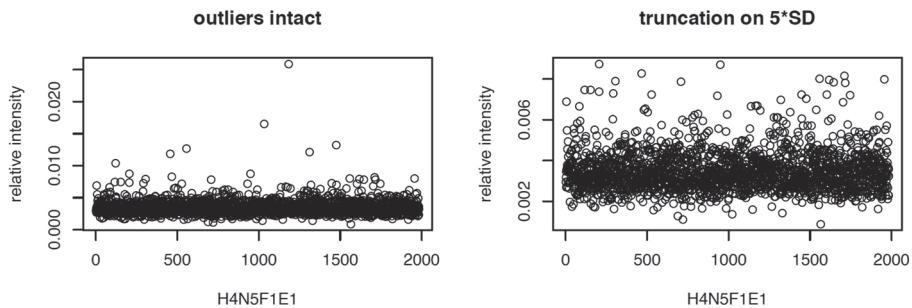


Figure 3: Outlier removal with a cut-off of 5x standard deviation

Figure 3 visualizes the effect of removing data outliers of one extracted analyte (H4N5F1E1) with a cut-off at 5x standard deviation from the mean. It is assumed that biological differences between the groups are smaller than $5 \times SD$ and that these outliers are due to technical artefacts during sample preparation or analysis.

A recent article expands the knowledge of blood protein *N*-glycosylation in a similar manner to **chapter 2**, highlighting the complexity of the *N*-glycome, its variation amongst different demographics, and its importance for understanding its variations in health and disease by comparing glycosylation changes in nine different diseases¹⁴. They showed that the glycosylation features found in IBD patients had

little resemblance to other diseases like breast, pancreatic and colorectal cancer or multiple myeloma while CD and UC exhibited rather similar glycosylation signatures (Figure 4).

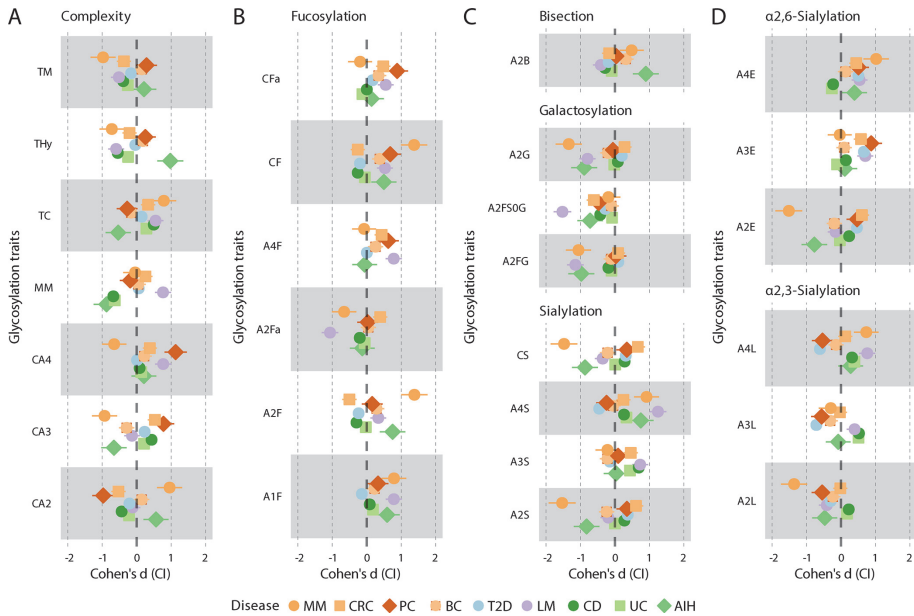


Figure 4: Forest plots visually illustrating the effect sizes of glycosylation traits observed in various diseases.

Taken from¹⁴. The data and results from the studies on CD and UC reported in this thesis⁴ was used by Pongracz et.al for comparison with the glycosylation changes generated on other diseases studied with similar methodological workflows and reported in their respective publications. Those studies were focused on multiple myeloma (MM)¹⁵, colorectal cancer (CRC)¹⁶, pancreatic cancer (PC)¹¹, breast cancer (BC)¹⁷, type-2 diabetes (T2D)¹⁸, leishmaniasis (LM)¹⁹ and autoimmune hepatitis (AIH)²⁰.

While these studies employed comparable methodologies, minor variations in sample preparation and data analysis may have introduced slight discrepancies in the results. Despite these differences, the most abundant glycosylation features (not the effect size or direction) remained consistent across all studies, whereas low-abundance analytes exhibited minor variability between individual investigations. Importantly, the use of glycosylation traits in all analyses effectively mitigated the impact of these inconsistencies. Importantly, glycosylation changes between healthy

controls and disease groups were consistently observed across studies, indicating that the cross-disease comparisons are valid and meaningful¹⁴.

In IBD, comprising CD and UC, patients show a significant reduction in IgG *N*-glycan galactosylation and sialylation, correlating with disease severity and inflammation. Increased core fucosylation suggests an immune-modulatory role, similar to Rheumatoid Arthritis (RA), where IgG shows high core fucosylation. Galactose-deficient IgG is a key inflammatory marker linked to disease activity and often precedes clinical symptoms²¹. Compared to other inflammatory diseases, CD and UC generally show similar glycosylation trends but rarely exhibit the most extreme changes, except for α 2,6-sialylation of tetra-antennary glycans (A4E) and α 2,3-sialylation of bi- and tri-antennary glycans (A2L, A3L). They also display a distinct profile of multiple mannosidic structures (MM), comparable only to autoimmune hepatitis, while most other diseases show little or no MM variation—except visceral leishmaniasis (LM), which exhibits a marked increase. Notably, IBD has a relatively mild inflammatory glycosylation profile, with less pronounced IgG degalactosylation in di-antennary glycans (A2G) than e.g. LM¹⁴.

IBD, including CD and UC, exhibit glycosylation changes that set it apart from other diseases, particularly cancers, autoimmune disorders, and metabolic conditions. Unlike Multiple Myeloma, which shows a strong skewing towards di-antennary glycosylation due to the secretion of large amounts of monoclonal immunoglobulins, IBD's glycosylation appear to involve IgG and IgA, with a consistent reduction in galactosylation and increase in sialylation. This pattern is indicative of chronic inflammation and shared with other inflammatory and autoimmune conditions, but distinct from cancer-related glycosylation alterations, which often involve increased branching (tri- and tetra-antennary glycans) and more enhanced sialylation. Pancreatic cancer (PC) and colorectal cancer (CRC) both exhibit a shift from di-antennary to more complex tri- and tetra-antennary glycans, alongside increased α 2,6-sialylation and fucosylation, whereas IBD does not demonstrate such pronounced branching or elevated sialylation¹⁴.

While both IBD and IgA Nephropathy (IgAN) involve altered IgA glycosylation, the signatures are distinct. IgAN is characterized by under-galactosylation of *O*-glycans in IgA1, which is thought to promote immune complex formation and kidney inflammation, whereas IBD shows more widespread glycosylation modifications affecting multiple glycoproteins in the circulation, including IgG and IgA. The systemic nature of IBD glycosylation alterations contrasts with the kidney-specific pathophysiology of IgAN. Similarly, autoimmune hepatitis (AIH) appeared to share some inflammatory glycosylation features with IBD, such as decreased galactosylation (more pronounced in AIH), but AIH also presents a marked increase in hybrid-type glycans and core fucosylation, a trait not prominently observed in IBD.

Moreover, AIH features a distinct reduction in di-antennary glycan sialylation and a significant decrease in oligomannosidic structures, whereas IBD's glycosylation changes are less pronounced in these areas¹⁴.

Interestingly, although IBD manifests in the gastrointestinal tract as does colorectal cancer, their glycosylation profiles are rather different. While CRC shows a characteristic increase in tri- and tetra-antennary glycans, unlike IBD, suggesting distinct systemic effects. Moreover, in contrast to IBD, CRC exhibits reduced core fucosylation of di-antennary glycans¹⁴.

Metabolic diseases such as Type 2 Diabetes (T2D) also share some glycosylation features with PC in terms of increased α 2,6-sialylation of tri- and tetra-antennary glycans, a feature that is not prominent in IBD. This reinforces the idea that IBD's glycosylation changes are more aligned with chronic inflammatory processes rather than metabolic dysregulation. Additionally, while infections such as visceral leishmaniasis (LM) can induce strong glycosylation changes, including a marked reduction in galactosylation of IgG, IBD presents a more moderate and chronic version of this inflammatory glycosylation pattern rather than an acute immune response¹⁴.

Overall, IBD's glycosylation signature is most similar to other inflammatory and autoimmune diseases, particularly in its reduction in galactosylation and sialylation, but it remains distinct from cancer-related glycosylation profiles that involve increased branching and sialylation. The ability of IBD glycosylation to predict treatment response, differentiates it from glycosylation signatures of other diseases¹.

In **chapter 4**, a new semi-automated sample preparation was developed to reduce benchtop errors during sample preparation for IgA capturing, digestion and glycopeptide purification and streamline glycopeptide analysis by reverse phase LC-MS. A previously published method described a workflow for capturing, purifying, and analyzing IgA glycopeptides using cotton-based HILIC extraction combined with MALDI-FTICR-MS²². Although this method provided excellent analytical performance, it was not easily scalable beyond a small number of samples due to the labor-intensive manual preparation of HILIC materials (cutting small cotton thread and immobilizing it inside pipette tips), the long pipetting sequence for the capturing and purification of the samples, and the complex data analysis required for MALDI-FTICR-MS datasets. In the research presented here, the core elements of the original method regarding specifically the IgA capture using camelid antibody domains immobilized on agarose beads and protein digestion with TPCK-treated trypsin are retained^{22, 23}. However, significant changes were introduced for the purification and data acquisition steps to enable high-throughput analysis of hundreds of samples. In

this optimized workflow, IgA glycoprotein purification is performed using 96-well filtration plates, replacing the manual HILIC pipette-tip approach. Furthermore, mass spectrometric analysis was transitioned to an LC-MS platform, which resolved prior challenges in simultaneously detecting high- and low-mass analytes that complicated analysis with MALDI-FTICR-MS^{22, 23}. To address the issue of sialic acid effects on glycopeptide retention in reverse-phase LC, optimized LC-MS conditions were developed by adding TFA to the eluents for the data acquisition, minimizing retention time shifts and enabling robust, high-throughput data processing, as described in two chapters of this thesis^{24, 25}.

Our novel approach for specific IgA glycosylation analysis provides detailed information on 69 measured IgA glycopeptides. Despite a small sample size limiting predictive model validation for IgAN, we demonstrated that IgA glycopeptides are better predictors of IgAN status and renal function than on lectin-based assays Gd-IgA levels and eGFR alone. Our findings suggest new opportunities for using glycopeptides as biomarkers for IgAN onset and progression.

After the publication of the manuscript presented in **chapter 4**, no other large glycomic studies have been performed in the context of IgA nephropathy with a similarly large number of glycans analyzed, most likely due to the technical challenges inherent to (*O*)-glycopeptide analysis mentioned in the discussion. This complicates the comparison of our findings with other research reports. A paper published one year after the manuscript of **chapter 4** was executed with a similar setup but based on an older protocol using desialylation of the *O*-glycans, allowing for simpler data analysis but lacking sialic acid information due to the neuraminidase treatment applied to their samples²⁶. Nevertheless, their research confirmed the findings reported in this thesis except the ones about SA that were out of the scope of their research.

The sample preparation protocol that was previously used for the analysis of IgA in our research group was executed manually, including the affinity purification, and the samples were analyzed by MALDI-FTICR-MS (Fourier transform ion cyclotron resonance)²². One advantage of this previous method was the high resolution obtained from the FTICR-MS instrument used allowing for easy and highly confident identification of the analytes, and the relatively small size of the data generated compared to the LC-MS data presented here. Some of the drawbacks from the previous protocol were that the sample purification had to be performed manually with many pipetting steps for the HILIC purification, and that the analytes had to be measured in two parts to optimize their detection with the FTICR-MS, and then normalized together for the rest of the analysis. The very high resolution of the analytes obtained from their research, including the MS/MS experiments performed with direct infusion ESI-FTICR-MS allowed us to use it as a basis for the research of

the analytes present in the IgAN study presented here. The other approach chosen in the study of **chapter 4 and 5** with the 96-well plate sample preparation allowed all samples to be prepared in a single campaign. For the data acquisition, the use of exclusively LC-ESI-MS(/MS) enabled the analysis of all glycopeptides in a single run, with the only drawback of the large size of files generated which required a dedicated computer and a series of hard-drives to be used to process the data at once. Overall, this approach greatly improved the workflow for the analysis of IgA glycopeptides and allows this protocol to be used again more efficiently in future studies.

One point that was not discussed in both the IgA papers was the comparison of the relative intensity of the glycopeptide clusters (HYT, LSL, TPL, LAG(C/Y), and MAG(C/Y)). The variability of ionization efficiency of each glycopeptide, the different time windows used for the extraction of each peptide clusters, the different charge states and different number of analytes in each peptide in addition to the differences of glycans detected in HC vs patients renders a biologically meaningful and insightful comparison of the sum intensities of the glycopeptide clusters level challenging due to the many uncontrolled variables. Once the challenges are mastered, assessing the ratios of glycopeptide clusters could for example be used for investigating if there is a change of IgA1/IgA2 ratio in disease. This could potentially replace additional assays for measuring immunoglobulin isotype and subclass concentrations. While calculating a ratio of each cluster based on the non-normalized intensity of each individual glycopeptide is technically possible, it would be difficult to defend its use as clinical marker due to the lack of a proper way to normalize all the intensities of the individual glycopeptides. The perfect way to remedy this would be the use of isotopically labeled glycopeptides for each analyte present in the samples, spiked as reference in each sample. However, the costs in time and money spent to produce such standards may possibly outweigh the benefits of having them. Another possible way to still have a constant standard analyzed with each glycopeptide clusters would be to add a single reference standard either in one of the buffers or in a very small amount (not to disrupt too much the ionization efficiency of the sample's analytes) in the nano-booster solvent during the ESI to avoid gradient effects due to buffer composition change during the elution of the analytes, and to use this as reference to normalize the intensity of each glycopeptide cluster, then enabling their comparison directly based on the LC-MS data.

In our study in **chapter 4 and 5**, we analyzed the *O*-glycopeptide of IgA1 containing multiple glycosylation sites as a single block. In theory, its analysis with site-specific information would have been possible to achieve, but with much more time needed to prepare the samples and do the multiple enzymatic digestions necessary to cleave the peptides around the individual glycosylation sites. This would not have allowed

the use of the robotized platform in the way we intended. A recent paper reporting on a technique based on sequential deglycosylation of the IgA hinge region with site-specific information about the order of most occupied Gd-IgA1 glycosylation sites, could potentially be used on a sample set in the context of IgAN but has currently only been tested with the plasma of ten healthy individuals²⁷.

When trying to explain the origin of the gd-IgA, multiple reports exist about glycosylation enzymes and altered gene coding sequences for galactosyltransferases^{28, 29}. Some heterogeneity in gd-IgA has also been observed amongst different populations although the glycosylation change with IgAN was similar in both populations³⁰. The reported changes in the expression of glycosylation enzymes may explain the lower galactosylation of IgA observed in our paper. However, if this was the main cause of gd-IgA and/or nephropathy, there should be reports for other proteins showing deficiencies in galactosylation associated with “IgA”N, which is currently not the case³¹⁻³⁴. A study investigated together the glycosylation changes of IgA and IgD in IgAN and while it confirmed the lower galactosylation of IgA, the contrary was observed for IgD, leaving more questions regarding the currently accepted explanation instead of confirming it. Although we have reported multiple glycosylation changes in the *N*-glycans of IgA1 and IgA2, most of the scientific reports seem to consider the *O*-glycosylation deficiency to be the main glycosylation cause of the harmful IgA complex formation, almost completely omitting the *N*-glycans from the equation³¹. However, these changes in *N*-glycosylation were found similarly in RA and IBD, giving hints that deregulated glycosylation may be shared between various inflammatory and autoimmune diseases.

The aberrant glycosylation of IgA appears central to the disease process, which leads to the formation of IgA self-aggregates which are then recognized by various ligands, triggering a cascade of inflammatory responses. Recent reports suggest that self-aggregation of IgA in Nephropathy patients could involve the cysteine-471 in the tailpiece of IgA1, although *O*-glycosylation deficiencies appear to promote nonspecific self-aggregation^{35, 36}. Some other reports suggest that initial IgA aggregates that are recognized by IgG, IgM and C3 are then later forming multi-IgA aggregates and that these are the ones depositing in the kidneys^{28, 30, 37}. It has been shown that the complement system can be triggered by multiple pathways in IgAN³⁸ and recent studies reports that the complement protein of the complement system plays a major role in the pathogenesis of IgAN, and that its presence in the IgA aggregates helps the diagnosis of IgAN from other glomerular IgA deposition^{39, 40}. As much as researchers agree that the deposition of IgA complexes in the kidneys are an important factor for the destructive immune response occurring in IgAN, the exact mechanisms of the disease still remain unclear⁴¹. Researchers compared the

serum levels of IgA and glomerular levels of secretory IgA (SIgA) in a biopsy sample from the kidney of a nephropathy patient, and found values 120 times higher for the SIgA, indicating a very high accumulations of SIgA, but not of monomeric IgA, in the glomerular deposits⁴². They also compared the binding affinity of monomeric and polymeric IgA (pIgA) to the mesangial cells and found a stronger binding of pIgA compared to monomeric IgA, supporting theories that abnormally glycosylated IgA, which has a tendency to self-aggregate, and IgA complexes being involved in the inflammatory activation chain in IgAN. Some other recent reports detail how the aberrant glycosylation of IgA affects its binding with some of its receptors, and when IgA receptors on cell membrane are under expressed in IgAN, can slow down the clearance of IgA from circulation and favorize the formation of the inflammatory complexes²⁹. A very interesting effect is also observed when IgA1 receptors like CD71 binding preferentially polymeric IgA1 (but not the monomeric IgA1/2), or integrin $\alpha 2/\beta 1$ are found to be overexpressed in the mesangial cells of IgAN patients and could be potential targets of new treatments^{29, 43, 44}.

In theory, any of these steps mentioned above could be the target of novel treatments for IgAN, although most of recent developments seem to focus on the mediation of the inflammatory reaction chain and ligands recognizing the IgA complexes^{45, 46}. Other current treatment candidates aim to reduce the production of pathogenic IgA in the gut or to target B cells to reduce the production of IgA, IgG and autoantibodies in general and to slow down the formation of the IgA-IgG-C3 complexes. There are also therapeutic strategies aimed at reducing the activation of the complement system, while others focus more on the mitigation of the effects by targeting a reduction of proteinuria to limit the damages to the kidneys and delay the progression of IgAN without actually treating the cause of the disease⁴⁷⁻⁵⁴.

Even if the perfect approach to treat and possibly cure IgAN has not yet been proposed, it is important to see that there is a growing interest in understanding the disease and that developing therapeutics with multiple modes of actions could be envisaged and maybe combined for better results.

With the research presented in the different chapters of this thesis, it was encountered that previous glycomic studies often used small sample sets and various in-house methods, making comparisons difficult. Large-scale analyses have previously been hampered by traditional sample preparation methods and the lack of dedicated software. This thesis uses larger sample sets with streamlined sample preparation, enabled by robotized platforms and new software for analyte extraction and statistical analysis. Automated liquid handling robots improved sample preparation consistency and reduced human error. More than a thousand samples were included in a single glycomic study, allowing powerful statistical corrections. In-house software was used to process large LC-MS datasets, reducing

them from terabytes to megabytes of analyte-focused information, ready for statistical analysis. The sample preparation techniques avoided biases associated with glycan labeling and used protein capture based on peptide portions, reducing analyte bias. Analyzing relative intensities per glycoprotein cluster removed variability in protein concentration without needing an internal standard. This approach, along with derived traits grouping similar features of analytes, provided stronger insights into global glycosylation changes in diseases.

Overall, the analytical workflows presented in this thesis show the growing potential of glycomic analysis in the development of non-invasive biomarkers and shines light on the potential of robotized workflows to accelerate the research for new biomarkers and offer clinicians alternative tools for early diagnosis, patient stratification and personalized medicine based on easily accessible samples^{12, 55-57}.

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