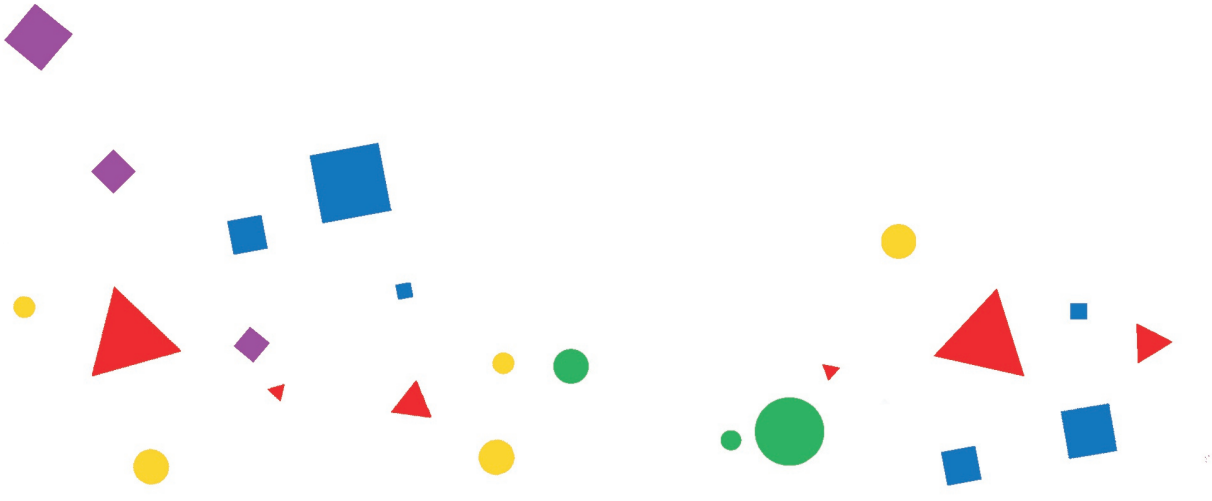
Glycosylation analysis of immune-related molecules

Borosak, I.



Chapter 7
Discussion and perspectives

7.1 MASS SPECTROMETRY IS OF PARAMOUNT IMPORTANCE IN GLYCOMICS DISCOVERY STUDIES

Mass spectrometry (MS) has emerged as a method of choice for clinical marker discovery studies. It provides precise mass information and structurally informative fragments with high sensitivity and specificity. Moreover, it enables the quantitative analysis of glycoform abnormalities. Clinical marker discovery only became practical with the introduction of high-throughput and robust mass spectrometry-based methodologies into glycomic studies.(1-3) Nowadays, a wide range of high-throughput analytical strategies is available for glycan-orientated clinical evaluation.(4, 5)

In this thesis, the glycan-orientated research studies were conducted using three types of mass spectrometers. In **Chapters 2** and **3**, IgG glycopeptides were analyzed by reversed-phase nano-liquid chromatography coupled to electrospray ionisation quadrupole time-of-flight mass spectrometry (RP-nLC-ESI-QTOF-MS). Finally, **in Chapter 5** and **6** hydrophilic interaction liquid chromatography hyphenated with electrospray ionisation time-of-flight mass spectrometry (HILIC-LC-ESI-TOF-MS) was utilized to analyze released N-glycans from either serum or seminal plasma. In **Chapter 4**, we employed reversed-phase nano liquid chromatography coupled to electrospray ionisation quadrupole Kingdon trap, commercially referred to as Orbitrap™ mass spectrometry (RP-nLC-ESI-KT-MS), to analyze FcγRIIIb glycopeptides. The glycopeptide workflow involved protein purification, enzymatic digestions, purification of glycopeptides, and their analysis by either RP-LC-ESI-TOF-MS or RP-LC-ESI-KT-MS. For the released N-glycan analysis, glycans were released from either a purified protein or total glycoproteins in the sample. Subsequently, the released N-glycans were labeled with a fluorescent tag, separated by HILIC-LC and analyzed by on-line ESI-TOF-MS. It is important to note, that each of the aforementioned LC-MS strategies is suitable for separating ions according to their mass-to-charge ratio, each with its own set of advantages and disadvantages in terms of mass resolution, accuracy, ion fragmentation capabilities, user-friendliness, throughput and level of structural information content.

The mass spectrometry instrumentation mentioned above involved ESI as a preferable soft ionization technique. Although not used in this thesis, there is another technique called matrix-assisted laser desorption/ionization (MALDI) widely used to ionize glycoforms. Both ESI-MS and MALDI-MS are attractive methods for clinical marker discovery on the level of released N-glycans, glycopeptides, as well as intact glycoproteins profiled from human biofluids.(6) They offer powerful and in-depth characterization of glycoforms with high-sensitivity and specificity. ESI-MS can be easily hyphenated with various separation techniques to further enhance the sensitivity, specificity and precision of the MS analysis. On the other hand, MALDI-MS includes a simpler sample preparation workflow that can be fully automated.(7, 8) Additionally, it eliminates the need for a separation system prior to

measurement, enhancing its throughput capacity, and making it a popular choice among researchers for high-throughput analysis.(8) However, limitation of MALDI-MS include the inability to separate glycan isomers, as well as the need for a derivatization strategy to preserve labile sialic acids.(8) Numerous studies have used these methods to identify altered glycosylation patterns in various diseases.(1, 9, 10) Mass spectrometry-based assays for glycan screening using either MALDI-MS or ESI-MS analysis are well-suited for clinical laboratories.(6, 11) However, despite the great potential, the clinical utility of these techniques is still in its early stages. Further research and development are needed to fully harness their power for clinical applications.

MS is an essential tool in glycan analysis, but it comes with inherent limitations. Since it relies solely on mass accuracy measurements, MS struggles to distinguish between structural isomers of glycoforms. The differentiation of isomers is important because distinct structural isomers can have varying biological relevance.(12, 13) To provide the capability to separate isomeric glycans and glycopeptides, MS is often coupled with various chromatography techniques, such as porous graphitized carbon, capillary electrophoresis, ion exchange, reverse phase (RP) and hydrophilic liquid chromatography (HILIC). Among these, HILIC and RP have shown great promise for high-throughput applications due to their robustness ad reproducibility, as demonstrated in the aforementioned studies. Hydrophilic interaction liquid chromatography (HILIC) is primarily used for separating released glycans.(14) Alternatively, HILIC can also be applied for glycopeptide analysis, resolving glycan linkage isomers. On the other hand, reverse-phase liquid chromatography (RP-LC) has become the most widely used method for glycopeptide separation.(15) RP-LC has likewise been shown to resolve glycan isomers and to be also employed for analyzing released glycans.(16) These chromatography techniques, when combined with MS offer a more comprehensive and versatile approach to glycan analysis, addressing challenges posed by structural isomers.

In **Chapter 3**, we employed a nLC-qTOF-MS to investigate the IgG glycosylation patterns of IgG in patients with ANCA-associated vasculitis (AAV). The results revealed specific glycomics markers that are closely linked to disease relapse. These findings contribute to the growing body of evidence indicating the significant role of glycosylation in maintaining overall health, and it association with the occurrence, progression and outcome of various diseases.(17-19) As a consequence, glycans have emerged as promising clinical markers with the potential to be utilized in disease diagnosis, prognosis and monitoring.(20) However, despite the rapidly growing body of evidence supporting the clinical value of glycan markers, there are challenges associated with the translation of the glycomic MS-based approaches into clinical laboratories.

The path from the discovery of a glycan marker to its successful clinical implementation involves a series of steps and requirements that must be fulfilled. The development of a method that reliably identifies a candidate clinical marker is just the initial phase in this process. Subsequent steps include the clinical validation of the marker and analytical validation of the method, demonstrating their sensitivity and specificity, obtaining regulatory agency approval, gaining acceptance from healthcare practitioners, and ultimately, their inclusion in appropriate medical guidelines. In the field of proteomics, a three-tier classification system (Tier 1, Tier 2, Tier 3) has been developed to outline the prerequisites for protein quantification assays in both protein biomarker discovery and well as their anticipated clinical integration.(21) These tiers represent the stringency of requirements for various assay performance metrics, including precision, accuracy, repeatability as well as the extend of analytical validation. As the tier decreases, the requirements become more stringent, indicating distinct characteristics of the analytical performance and preferred applications for each tier. Specifically, Tier 3 methods are primarily used for clinical markers discovery, Tier 2 for clinical marker quantification and validation, and Tier 1 for clinical implementation. To facilitate the transfer and implementation of quantitative MS-based glycomics assays across different clinical laboratories, glycan and glycopeptide markers could be subjected to standardized strategies similar to those developed for protein markers. A comparable Tier 1 for glycan and glycopeptide markers would necessitate absolute quantification through the use of stable isotope-labeled (SIL) internal standards. Indeed, the incorporation of stable isotope-labeled internal standards into glycans and glycopeptides methods would enable improved absolute quantification of individual glycoforms in a standardized manner, facilitating the establishment of consistent reference ranges for diagnostic purposes.(22). However, the current absence of well-established synthetic SIL analogs for released glycans, glycopeptides, and intact glycoproteins impedes the translation of glycomics and glycoproteomics profiling into standardized clinical assays. Consequently, most MS-based glycomic approaches are currently better suited for clinical marker discovery studies, where relative quantification of individual glycoforms in relation to the total glycoform signal intensity is deemed acceptable.(6) Nonetheless, the growing interest in clinical glycoscience will drive further advancements in the development of internal standards necessary to facilitate the translation of these approaches to clinical laboratories.(23) Alternatively, the clinical translation of glycan-based markers can be facilitated through the implementation of proposed tiers tailored for glycan-multimarker panels, especially with the regard to quantification.(24)

The analytical methods anticipated to translate clinical markers from discovery studies to clinical practice must deliver excellent diagnostic performance and undergo rigorous validation. Additionally, these methods should be fit-for-purpose, robust and cost-effective. Ideally, these methods should be straightforward enough for healthcare providers to

perform. While mass spectrometry offers numerous advantages in discovery glycomics analysis, it also comes with completely opposite characteristics, such as high operational costs, complex sample preparation procedures and the requirement for sophisticated instrumentation and skillful personnel, particularly when analyzing glycans. One elegant solution to this challenge is to simplify the analysis approach and shift away from MS-based methods which are used for clinical marker discovery, towards non-MS-based platforms for clinical deployment.(25) By transitioning to non-MS-based platforms, particularly immunoassays and plate-based assays, the barriers associated with cost, complexity and expertise can be overcome, making the glycan-based clinical marker analysis more accessible. When established with a high level of accuracy and precision, these non-MS-based platforms have the potential to be easily and successfully implemented in existing molecular diagnostic laboratories. The transition of glycomic assays between platforms is exemplified by the development of an immunoassay for relative and absolute quantification of antigen-specific IgG fucosylation, which serves as a clinically relevant marker for altered IgG effector functions, such as antibody-depended cellular cytotoxicity.(26) Initially, LC-MS-based methods were used to characterize antigen-specific IgG Fc-fucosylation in alloimmune diseases or viral infections (27, 28) Subsequently, a fucose-sensitive ELISA-based method for quantifying antigen-specific IgG (FEASI) was developed for assay validation and potential clinical utilization. FEASI is a high-throughput assay that can be implemented in diagnostic laboratories, for example, for screening patients with lower IgG Fc fucosylation on anti-S antibodies, which has the potential to guide the treatment of conditions such as COVID-19.(26) This approach eliminates the need for sophisticated equipment and complex analytical and computational processing required for antigen-specific IgG Fc glycosylation analysis. While plate-based glycomics assays are sensitive and relatively costs-effective, and therefore well suited for the clinical laboratory, they are challenged by technological limitations. These limitations include cross-reactivity, limited specificity, interference and low multiplexing capability. This becomes crucial as often multiple glycosylation features, need to be analyzed simultaneously as a panel of structures rather than individually. However, with the current availability of novel antigen preparations (recombinant, highly purified proteins, synthetic oligosaccharides) along with other methodological improvements, a new generation of immunoassay-based and plate-based methods is being developed, enabling the investigation of glycan structures even in minute amounts of antigen-specific responses.(26, 29)

Immunoassays are one of the main diagnostics tools in today's clinical laboratories, offering several distinct advantages. They stand out primarily for their minimal sample preparation requirements and the absence of the need for sophisticated instrumentation. Instead, immunoassays rely on straightforward detection methods, such as photo-, fluoro-, or luminometric techniques, replacing chromatographic approaches in clinical diagnostics. These methods benefit from simplicity, rapidity, sensitivity, and potential for automation,

making them less dependent on the operator's skill. For example, the measurement of the carbohydrate antigen 19-9 (CA 19-9), also known as sialyl-Lewis[a] antigen, is commonly conducted using immunoassays.(30, 31) Within clinical laboratories, a range of immunoassay techniques, including electrochemiluminescence immunoassay, enzyme immunoassay or microparticle-enhanced enzyme immunoassay, is employed to assess elevated CA 19-9 levels in circulation.(32) Notably, elevated CA 19-9 levels are indicative of pancreatic cancer and are instrumental in monitoring the effectiveness of therapy.(33)

7.2 GLYCAN SIGNATURES AS POTENTIAL CLINICAL MARKERS

Glycomics and glycoproteomics have emerged as promising sources of clinical markers, and this expansion shows no sign of slowing down as new technologies come into play.(24, 34, 35) Among the most extensively studied glycan markers are the glycosylation of total plasma proteins (plasma total N-glycome, TPNG) and the glycosylation of the most abundant plasma glycoprotein, immunoglobulin G (IgG N-glycome).(18, 36) Both plasma and IgG N-glycans can offer valuable insight, for example distinguishing between states of health and disease, discerning different disease subtypes or predicting disease outcomes. The growing availability of data from retrospective cohorts, as exemplified in **Chapters 2, 3**, and **6,** has led to significant advances in our comprehension of how alterations in N-glycosylation are associated with the onset, progression and severity of various diseases, shedding light on pathophysiological processes. Notably, as demonstrated in **Chapter 3,** glycans can be tracked longitudinally to monitor treatment response and predict the likelihood of disease relapse. Glycan markers are explored for multiple clinical purposes, including diagnosis, prediction, prognosis, guiding treatment decisions, and assessing treatment response.(17, 24)

7.2.1 Plasma glycan-based clinical markers

Plasma serves not only as the primary clinical specimen but also as a readily accessible source for the discovery of novel clinical markers.(18, 37) Given that the vast majority of plasma proteins undergo glycosylation, exploring plasma protein N-glycosylation holds enormous potential for identifying specific glycosylation signatures associated with a wide range of diseases.(18) **Chapter 5** describes an optimization of a novel profiling approach for total plasma N-glycome. This method utilizes user-friendly LC-MS instrumentation and a novel fluorescent label with an *N*-hydroxysuccinimide carbamate tag, quinolone fluorophore, and tertiary amine to enhance ionisation efficiency.(38) With its simplicity and robustness, the method opens exciting avenues for translating glycomic profiling into clinical practise.

Profiling of total plasma N-glycans, encompassing the release and analysis of N-glycans bound to all plasma proteins, and provides data on alterations in glycan structures. Changes in plasma protein glycosylation constitute an important phenotypic feature of both health

and disease and hold potential as differential clinical markers. The vast majority of glycoproteins in plasma, including acute phase proteins and apolipoproteins, originate from liver. Therefore, it does not come as a surprise that these plasma glycosylation patterns predominantly mirror liver (patho)physiology.(37, 39) The differences in the level of bisection and galactosylation are indicative of an individual's liver health status, and methods quantifying these differences are currently being applied for the differential diagnosis of liver fibrosis.(40). This success story exemplifies a plasma glycomics-based test, readily available for clinical use.

In addition to its relevance in liver disorders, the plasma N-glycome holds the potential for differential diagnosis of pathologies arising from mutations in genes which are implicated or directly involved in glycosylation processes. This includes conditions such as congenital disorder of glycosylation (CDG) and maturity-onset diabetes of the young type 3 (MODY3). Notably, disease-causing mutations in these diseases exert systemic effects on the glycosylation processes. MODY3, a subtype of maturity-onset diabetes of the young, is caused by mutations in the *HNF1A* gene, which encodes hepatocyte nuclear factor-1α (HNF1α). HNF1α regulates the expression of several liver-specific genes, including those encoding fucosyltransferases.(41) Genome-wide association studies (GWAS) showed that the lower activity of HNF1A leads to the downregulation of α1-3 and α1-4 fucosyltransferase. Subsequent studies proposed a reduction in antennary fucosylation levels of plasma N-glycans as a hallmark of HNF1A-MODY.(42) Subsequently, the antennary fucosylation signature of plasma proteins demonstrated high differential diagnostic performance in distinguishing HNF1A-MODY using glycomics workflows. (43) A novel exoglycosidase plate-based assay, has been developed for the quantification of antennary fucosylation markers, offering a straightforward diagnostic tool.(29) This assay paves the way toward integrating N-glycomic approaches into clinical practice for the diagnosis of individuals with deleterious HNF1A variants.

In the context of CDG diagnosis, the exploration of plasma N-glycan differences shows promise for improving the diagnostic process. CDG are rare inherited conditions caused by recessive mutation that affect protein N-glycosylation. Currently, CDG diagnostic relies on carbohydrate-deficient transferrin (CDT) profiling, typically by isoelectric focusing. However, CDT has limited diagnostic value. For instance, in cases of SLC35A2-CDG, which are caused by genetic defects in the uridine diphosphate (UDP)-galactose transporter, classical transferrin analysis often fails to detect the disease due to an unchanged transferrin glycosylation profile in the majority of patients. As a result, researchers have broadened their focus and look at plasma N-glycans, which encompass alterations in the glycosylation of the most abundant glycoproteins.(44) Recently, Chen et al. identified potential new N-glycan markers by employing a fast and semi-quantitative MS-based technique using flow-injection ESI-QTOF.(45) They pinpointed oligomannose N-glycans as

potent clinical markers for screening different CDG subtypes. Moreover, by making use of an isotope-labeled sialylated glycopeptide they were able to semi-quantitatively assess N-glycans. Their approach proved efficient for the diagnosis of certain CDGs and demonstrated superiority over alternative methods, particularly in terms of robustness and turnaround time.

Although very promising in detecting some genetic diseases, a wide variety of diseases associate with similar changes in plasma protein glycosylation. Generally, an aberrant plasma N-glycome such as an increase in fucosylation, branching or sialylation, has been cross-sectionally linked to numerous pathological states. These range from diabetes, and inflammation to even cancer.(18, 46) Thus, specificity and sensitivity as a stand-alone clinical marker are missing. Moreover, total plasma N-glycan profiling is biased by the variation in protein concentration. When studying total plasma N-glycome with glycomics methods, we are not able to determine if the observed glycosylation changes originate from variations in protein concentration and/or variations in protein glycosylation.

To eliminate this variation and to increase the sensitivity and specificity of glycan-based markers derived from plasma, an approach that involves protein-specific and site-specific glycosylation analysis is being explored. This method includes the isolation of the protein of interest and the analysis of both the protein backbone and the attached glycans. By specifically targeting the glycosylation patterns of certain plasma glycoproteins, such as alpha-fetoprotein (AFP), there is great promise for improving the accuracy of differential diagnosis. AFP is a plasma marker for diagnosing hepatocellular carcinoma (HCC). However, its performance in early HCC detection is limited by low specificity, especially in patients with benign liver disease. In light of this limitation, a particular proteoform of AFP, fucosylated alpha-fetoprotein-L3 (AFP-L3) has proved to be more specific and sensitive, making it a promising new generation HCC marker that allows for earlier diagnosis, assessment of therapeutic effects and evaluation of the biological malignancy.(47, 48) The test, which evaluates both the percentage of AFP-L3 and the AFP concentration is now FDA-approved for use as a marker for determining the risk of HCC in clinical settings. By delving deeper into protein-specific glycosylation analysis and exploring specific proteoforms like AFP-L3, we are making substantial progress in developing glycan-based clinical markers for targeted disorders. This advancement holds potential for disease diagnosis and personalized medicine, ultimately leading to improved patients outcomes.

Another way to increase the specificity of plasma-derived markers is to conduct site- and protein-specific analysis of glycosylation, for instance, based on intact glycopeptide workflows. The ideal scenario would entail the simultaneous site-specific examination of protein glycosylation across total plasma proteins using a multiplexed analysis method. Recent efforts have been directed toward optimising the analysis of a large number of

glycopeptides within a single glycoproteomics workflow for plasma samples, using different tandem mass spectrometry approaches.(49) As such, individual glycopeptide differences can serve as highly specific signatures, thus improving the specificity of plasma glycans and their clinical utility in monitoring and improving CDG diagnostics.(49) Insights gained from this innovative large-scale site-specific glycosylation analysis provide an exciting perspective toward understanding disease processes, and designing individualized diagnostics based on glycosylation status. Nevertheless, a current challenge lies in the availability of bioinformatic tools capable of handling the extensive and intricate site-specific glycosylation data and correlating meaningful glycoproteomic changes with clinical phenotypes, thereby ensuring its practical clinical application.

7.2.2 Immunoglobulin G glycosylation-based clinical markers

N-glycans of immunoglobulin G, the most abundant immunoglobulin in human plasma and a key component of the adaptive immune response, have emerged as potential clinical markers. Years of research in profiling IgG glycosylation revealed alterations in IgG glycosylation profiles in various clinical conditions, enabling differentiation between healthy controls and individuals with disease, patient stratification and disease prediction.(20, 36) For instance, profiling of IgG glycosylation has shown promise in distinguishing early-stage thyroid cancer from non-cancer controls by utilizing several N-glycan structures as differential markers. (50)

While glycan-based markers demonstrate high accuracy in disease detection, they often lack sensitivity, leading to a risk of false-positive findings. This limitation is particularly concerning when using IgG N-glycans as a stand-alone clinical marker. Many disparate diseases very often exhibit similar IgG N-glycan trends, showing changes in galactose, sialic acid, fucose and bisected GlcNAc content that move in the same direction. Consequently, changes in total IgG glycosylation do not appear to be disease-specific but rather represent non-specific effects, reflecting a general immune activation or common underlying inflammatory mechanisms.(51)

Despite the limitations of IgG N-glycans as stand-alone clinical markers in clinical practice, their integration with other diagnostic measures can improve diagnostic sensitivity and specificity, addressing clinical needs. For instance, a promising complementary analysis involves the combination of glycoprotein cancer antigen 125 (CA-125), an FDA-approved serum clinical marker for ovarian cancer detection, with IgG N-glycans. While CA-125 is frequently used, its performance in diagnosing ovarian cancer is only modest due to poor specificity, as it often increases in benign gynaecological pathologies. However, incorporating the quantification of IgG (a)galactosylation, which has been found to differentiate between benign and malignant stages, in multiplex clinical marker platforms improves the specificity from 62.2% to 84.6%.(52) This combined approach refines the

differential diagnosis of ovarian cancer, highlighting the importance of combining clinical markers in future diagnostic and prognostic strategies. A similar strategy to enhance the robustness of existing clinical markers is demonstrated in **Chapter 3**, where the performance of an ANCA rise for detecting AAV relapse could potentially be improved by considering IgG fucosylation. Given the complex nature of various disease responses and the heterogeneity of patient responses, combinations or panels of clinical markers have the highest potential to become a key aspect of future diagnostic and prognostic strategies. However, further research is needed to determine the most effective panels for each purpose. Additionally, to enable this shift, technology needs to be developed that allows for the simultaneous assessment of multiple markers from a single blood sample.

While this thesis is focused on the evaluation of longitudinal and cross-sectional alterations in total IgG glycosylation, another very promising approach to improving the specificity of glycan-based clinical markers involves isolating and analysing the glycosylation of antibodies against specific antigens. Especially those produced in response to infectious agents, as well as those playing a central role in the pathogenesis of autoimmune and alloimmune diseases are interesting targets. Profiling the glycosylation of these antigen-specific antibodies not only holds the potential to predict disease activity, but also provides valuable insights into the immunological response and the underlying mechanisms driving the pathologies. By focusing on these specific antibodies, we can gain a deeper understanding of disease progression and the factors influencing it. Indeed, in many cases of infectious, autoimmune and alloimmune diseases, the N-glycosylation of these antigen-specific antibodies has been reported to be altered and different from the total IgG.(28, 53, 54) Specifically, IgG Fc-fucosylation has emerged as a critical pathological feature in infectious diseases and alloimmune diseases.(55) Mechanistically, the low core fucose content on IgG amplifies affinity to Fc-gamma receptor IIIa (FcγRIIIa) and IIIb (FcγRIIIb), thereby increasing downstream signalling and escalating antibody-depended cell-mediated cytotoxicity (ADCC). These changes can profoundly impact the effector functions of IgG and trigger pro-inflammatory responses of the immune system, ultimately leading to disease exacerbation. Recent studies have pointed towards low fucosylation levels on antigen-specific as a hallmark of severe disease and demonstrated their potential to stratify patients upon diagnosis of coronavirus disease 2019 (COVID-19) infection.(56) Consequently, future tests monitoring antigen-specific IgG N-glycosylation features can aid in patient stratification and assessing the effectiveness of specific treatments. This can potentially lead to adjustments or alterations in therapy to maximise the beneficial effect on the patients' health.

The integration of established clinical markers like proteins and cytokines with newer omics markers, such as glycans, is both is exciting and promising. Glyomics-based clinical markers have the potential to advance personalized medicine significantly. Notably, IgG glycosylation cannot only aid in diagnosing diseases but also predict the disease

development as well as the risk of developing certain conditions.(36) However, there are still many challenges to overcome before moving from the discovery phase to clinical use. The current limitations of the field include 1) the lack of an internal standard enabling absolute quantification and control for technical variability; 2) the need for validation of the primary results on the larger population in multicentre analysis and prospective cohorts; 3) the development of assays that can be easily transferred to clinical settings. To unlock the full potential of IgG N-glycomics and make significant strides in discovery and innovation, further research is required to address these limitations. By doing so, we can pave the way for incorporating glycomics into clinical practice, ultimately improving patient outcomes and advancing personalized medicine.

7.3 FcγRIIIb GLYCOSYLATION AND ITS FUNCTIONAL RELEVANCE

IgG antibodies play a pivotal role in triggering antigen-specific immune responses by binding to FcγRs located on the surface of various effector cells. This binding event initiates a cascade of downstream effector signalling events. For example, in human neutrophils, the interaction of IgG with FcγRs can trigger processes such as phagocytosis, NET formation, and degranulation.(57) The strength of this immunologically relevant IgG-FcγR interaction is profoundly influenced by the proteoform distribution of both binding partners. Notably, N-glycosylation of not only IgG but also FcγR has been found to modulate the binding affinity between IgG and FcγR.(58, 59) Despite the significance of glycosylation in this context, comprehensive profiling of endogenous FcγR was limited. These constraints arise from various analytical challenges, including the scarcity of endogenous FcγR material, substantial structural diversity of glycans, and the presence of multiple glycosylation sites in close proximity.

In an effort to address this issue, **Chapter 4** outlines the analytical method designed to enable the straightforward and comprehensive site-specific profiling of vastly understudied N-glycosylation of FcγRIIIb isolated from a primary human cell material from a single donor. While other research groups have also developed analytical methods for site-specific glycosylation profiling of FcγRIIIb, our analytical approach overcomes several limitations of prior studies.(60-62) In particular, it allowed for the individual assessment of all glycosylation sites across multiple donors in a single LC-MS run. The results revealed an extensive compositional heterogeneity, spanning from oligomannosidic structures to complex sialylated tetra-antennary glycans with LacNAc extensions. While this method has allowed for a comprehensive and in-depth characterization of glycopeptides structures, uncovering the intricate details of glycan structures still remains a challenge. The study would benefit from an orthogonal method addressing for instance, isomer separation. Recently, progress has been made and new LC-MS/MS-based workflows showed the capability to determine glycan structural isomerism at the glycopeptide level, thereby achieving a higher degree of isomer separation.(63)

Furthermore, by incorporating several modifications into our method, it has the potential not only to improve isomer separation resolution but also to become a sensitive, robust and powerful workflow for high-throughput FcγRIII glycopeptide analysis. For instance, the initial glycopeptide identification was performed using the Byonic software. A substantial part of the process involved manual interpretation of both MS and MS/MS data. While effective for smaller sets, this approach becomes progressively more time-consuming and less efficient when analysing larger sample sets. Recent advancements in bioinformatic tools have partially addressed this challenge through the use of GlycopeptideGraphMS, which streamlines glycopeptide identification, curation and quantification following LC-MS(/MS) analysis.(64, 65) The manual identification process can now be semi-automated by integrating multiple software packages, such as Byonic, GlycopeptideGraphMS and LaCyTools. This integrated approach would reduce the time and effort required for manual analysis, thereby enabling the efficient processing of larger and more complex data sets.

There are also several unexplored aspects regarding the functional and clinical impact of FcγRIII glycosylation. Despite recent studies implicating the active role of glycosylation of the FcγRIII in the interaction with IgG,(58) there remains a notable scarcity of information regarding the glycosylation profile of the receptor in both healthy and diseased states. The potential application of this method to clinical samples would enhance our understanding of the clinical significance of differential FcγRIII glycosylation in healthy and diseased individuals. One compelling example is ANCA-associated vasculitis, a condition where the neutrophil activation plays a key role in damaging surrounding vessels and thus contributing to the disease mechanism. Exploring differences in FcγRIII glycosylation between healthy and diseased individuals represents a fascinating avenue for further research.

One key question pertains to the dynamics of N-glycosylation in FcγRIII. It raises the intriguing question whether potential glycosylation modulation is one the mechanisms by which neutrophils regulate their FcγR-depended functions. For now, the data indicate that *N*-glycosylation of neutrophil-derived FcγRIIIb is rather consistent between healthy individuals. However, it significantly differs from the profiles of soluble FcγRIIIb, and that of recombinant or serum-derived and NK cell-derived FcγRIIIa profiles.(60, 64) This provides very interesting perspectives on the dynamic changes of FcγRIII upon activation and thus ground for future studies on the interaction between FcγRIIIb and IgG.

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