Glycosylation analysis of immune-related molecules

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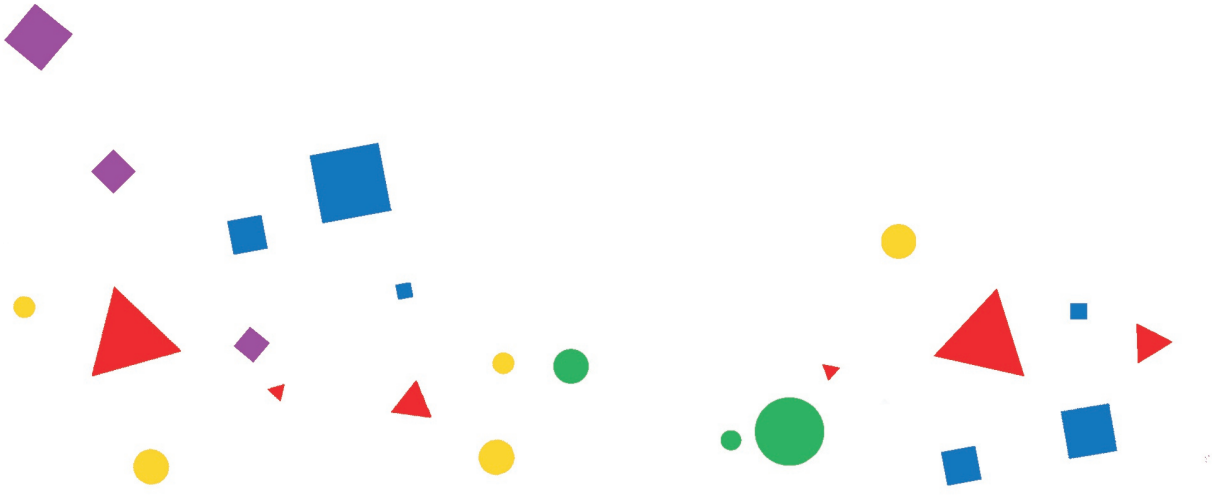

Addendum

English Summary

Nederlandse Samenvatting

Curriculum Vitae

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Acknowledgments

ENGLISH SUMMARY

The immune system comprises a complex network of organs, tissues and cells with various types of biomolecules of which glycoproteins are an important class. Glycoforms are variants of a specific glycoprotein (coded by a single gene) that differ in the composition and/or structure of the attached glycans. Moreover, the site-occupancy of each glycoprotein may be different. Glycans on immune molecules modulate protein structure and function, thereby facilitating cell-cell recognition, signal transduction and interactions with other glycoproteins or the extracellular matrix. In this manner, glycans play a vital role in initiating and regulating both adaptive and innate immune responses. Changes in protein glycosylation may originate from genetic, epigenetic or environmental factors and resulting aberrances can impact immune function and are associated with immunopathological processes. For example, it is known that changes in immunoglobulin G (IgG) glycosylation influence effector mechanisms such as complement activation and antibody-dependent cellular cytotoxicity. It is therefore important to develop methods that provide a detailed insight into protein glycosylation in order to gain a comprehensive understanding of key immune-related glycoproteins, and translate these findings into clinical applications. This knowledge could aid in deciphering immune-related processes in health and disease, establishing clinical markers for early disease detection, and optimizing the glycosylation of biopharmaceuticals for enhanced therapeutic benefits.

In this thesis the intricate associations between glycosylation on immune-related molecules and clinical features across diverse biological contexts are discussed. By integrating cutting-edge liquid chromatography (LC) and mass spectrometry (MS) methodologies for N-glycan analysis, the research described in this thesis revealed glycan signatures of antibodies in two population studies, but also extended this type of methodology to other proteins (Fc gamma receptors; FcγRs) and matrices (semen). Through a comprehensive analysis of glycosylation patterns, structural determinants, and pathophysiological changes, the thesis revealed new associations underpinning glycan-mediated immune modulation.

In **Chapter 1**, the significance of protein N-glycosylation in the immune system is discussed, with a particular focus on the influence of various proteoforms of IgG and FcγRs on immune responses. N-glycosylation, intricately regulated by cellular metabolic states and environmental conditions, is crucial for various immunological processes such as intrinsic and extrinsic recognition mechanisms, signal transduction pathways and effector functions. Alterations in glycosylation patterns profoundly impact fundamental immunological processes and their association with inflammatory and autoimmune diseases. Furthermore, in this chapter, advances in MS technology are discussed that enable the identification of disease-specific glycan signatures, followed by highlighting potential clinical implications of aberrant glycosylation of immune glycoproteins as markers for disease diagnosis and prognosis.

Regulation as well as function of IgG Fc-glycosylation is still not fully understood, despite substantial studies that have included various clinical conditions. **Chapter 2** endeavours to

address this gap by investigating the role of the spleen in IgG glycosylation. Utilizing a well-established LC-MS glycoproteomic workflow, it examines the effect of splenectomy on IgG Fc-glycopeptides sourced from plasma samples of individuals who underwent splenectomy due to trauma (n = 38), immune thrombocytopenia (ITP, n = 35), or spherocytosis (n =16) in comparison to controls with intact spleens (n = 165). The findings revealed that splenectomy led to an increase in IgG1 and IgG2/3 fucosylation, particularly pronounced in trauma and ITP cases. Furthermore, ITP patients exhibited lower IgG1 fucosylation compared to healthy controls, suggesting an altered immune response in this autoimmune condition. Overall, the findings indicate the important role of the spleen in formation and maintenance of IgG afucosylated responses, with implications for autoimmune diseases and immune responses to infections. Further research is needed to understand the mechanisms underlying these changes and their clinical implications.

One aspect of using antibody glycosylation in a clinical application is the ability to discern diseased individuals from healthy ones. Alternatively, and equally vital, antibody glycosylation provides insight into specific disease types or the likelihood of disease relapse. This offers the opportunity for personalized monitoring of a patient's disease progression and response to treatment. In **Chapter 3** the potential of IgG Fc-glycosylation as prognostic marker for relapse prediction in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is described. In this study, serum samples from 89 AAV patients were collected at up to six time points spanning from diagnosis to relapse or time-matched remission. Total IgG glycosylation was determined by LC-MS with a particular focus on its longitudinal dynamics during initial treatment, maintenance therapy, follow-up and relapse. The results revealed differences in IgG Fc-sialylation and bisection between relapsing and non-relapsing patients. The reduction in IgG sialylation was observed months before relapse, underscoring its utility as a relapse marker. While sialylation differed in the year ahead of the relapse, bisection already differed at the time of diagnosis between relapsing and non-relapsing patients and correlated with long-term treatment efficacy. Importantly, the differences in IgG fucosylation between relapsing patients and non-relapsing provided compelling evidence of orthogonality of ANCA-rise and IgG fucosylation, underlining the potential of combining these markers aiming to improve patient outcomes.

So far many glycosylation studies focused on IgG. This can be partially attributed to the high abundance of IgG in the blood and the relatively low complexity of its glycosylation, which simplifies the analysis. However, in the context of initiation and regulation of immune effector responses, the glycosylation of not only IgG, but also its respective receptors is crucial, such as Fc-receptor FcγRIIIb which is found exclusively on granulocytes. In **Chapter 4**, a novel glycoproteomic method for site-specific profiling of FcγRIIIb with four to six potential N-glycosylation sites is introduced, addressing previous methodological limitations. The methodology involves the isolation of FcγRIIIb from primary human neutrophils and subjecting it to in-gel digestion followed LC-MS/MS analysis. This approach allows for simultaneous mapping of almost all glycosylation sites in a single experiment,

providing detailed insights into glycosylation site occupancy, glycan composition, and structure. Results demonstrated the complexity of FcγRIIIb glycosylation, revealing site-specific variations in glycan composition and occupancy. For example, distinct glycosylation sites varied in positional isomerism and abundance of fucose residue. Specifically, site N45 exhibited solely core fucosylation, whereas sites N74 and N169 predominantly displayed antenna fucosylation. Additionally, site N162 demonstrated a concurrent presence of both core and antenna fucosylation. Moreover, the study identifies two previously unexplored glycosylation sites, N74 and N169, and elucidates differences between FcγRIIIb glycosylation profiles in neutrophils compared to its soluble form and the homologous FcγRIIIa. Neutrophil-bound and soluble FcγRIIIb differed in the occupancy of site N64. Specifically, in the neutrophil-bound form, site N64 was unoccupied, while in the soluble form, highly branched glycans were observed at this site. A major difference between cell-bound FcγRIIIb and FcγRIIIa was noted in the level of antennary fucosylation. Namely, FcγRIIIb exhibited a higher degree of antennary fucosylation at sites N74, N162 and N169.The findings highlight the importance of glycosylation in modulating FcγRIIIb function which may be implicated in antibody-mediated immune responses. Moreover, the presented methodology offers a robust platform for future investigations into the role of FcγRIIIb glycosylation in various physiological and pathological contexts, with implications for therapeutic interventions and personalized medicine.

Various workflows are currently employed for high-throughput N-glycan analysis, allowing for the identification and characterization of protein N-glycosylation at different levels. This can include the characterization of glycopeptides, as described in preceding chapters, or released glycans, as shown in the subsequent chapter. While methodologies for released N-glycan analysis continue to evolve, new advancements are consistently emerging. One such innovation applies RapiFluor-MS, a fluorescent tag incorporating a quinoline fluorophore and a tertiary amine. This tag simplifies the sample preparation process and enhances fluorescence and MS detection. **Chapter 5** details on the development and optimization of a hydrophilic interaction LC-MS method for the analysis of RapiFluor-MS labeled N-glycans from plasma and serum samples. The optimized method effectively profiles and separates N-glycans into 44 individual glycan peaks with 72 assigned compositions. Moreover, the method was applied to assess glycosylation in a population cohort, demonstrating its capability to detect biological variability of the serum N-glycome and suggesting its utility for biomarker discovery and biopharmaceutical development.

Next to IgG N-glycosylation analysis, the field of glycomics has largely expanded with regard to plasma or serum N-glycome analyses. N-glycome analysis can also be applied to alternative biofluids, such as seminal plasma, and was used to study the potential correlation of N-glycosylation with sperm function, semen abnormalities and male reproductive system disorders. Factors such as hormonal imbalances, genetic aberrations, and lifestyle habits, including smoking, alcohol consumption, and exposure to environmental and occupational hazards contribute to loss of sperm DNA integrity and male

infertility. However, there is a limited understanding of the association between seminal plasma N-glycome, semen DNA fragmentation (SDF) and environmental exposure in men. In **Chapter 6** these associations were explored among men with normal (n = 82) and abnormal semen parameters (n = 84). In subjects with normal semen parameters, significant and simultaneous associations of sialylated N-glycans eluting in SPGP5 ($p = 0.006$), SPGP17 ($p = 0.025$), and SPGP26 ($p = 0.038$) with SDF, exposure to photocopying and smoking were observed. In subjects with abnormal semen parameters, a significant simultaneous association of diantennary digalactosylated N-glycan with core and antennary fucose eluting in SPGP18 with SDF ($p = 0.001$), and smoking was detected. The study concludes that seminal plasma N-glycan profile disturbances are associated with reduced semen quality and exposure to environmental factors. The findings offer new insights into male infertility risk assessment and underscore the potential of N-glycans as clinical markers for exposure to environmental stressors, but also highlight the need for further research to validate these findings.

Chapter 7, the general discussion, concludes this thesis. The performed research is contextualized within the existing literature. The strengths of MS-based glycomics methodology for clinical marker discovery studies are discussed, such as high-throughput and high-sensitivity, as well as the challenges with regard to separating structural isomers. Following this, it critically evaluates the feasibility of transferring and implementing these approaches into clinical practise. In this chapter non-MS platforms are presented as potentially simpler and more cost-effective alternatives, thus enhancing the potential for successful implementation in clinical settings. Lastly, the chapter discusses the potential of plasma-derived glycan-based clinical markers in the realm of personalized medicine, emphasizing the importance of integrating these markers with existing ones into multifactor platforms to enhance sensitivity and specificity.