Glycoproteomics characterization of immunoglobulins in health and disease

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Summary (English)

Glycosylation of immunoglobulins is suspected to play a key role in the regulation of the immune system. Glycosylation of IgG has been shown to influence various effector functions, while IgE glycosylation is essential for receptor binding. Furthermore, various diseases have been associated with aberrant Ig glycosylation profiles – for instance, low galactosylation and sialylation of IgG *N*-glycans in autoimmune disorders, and low galactosylation of IgA *O*-glycans in IgA nephropathy.

Glycans can occupy multiple positions within Igs: each heavy chain harbors at least one *N*-glycan, which can affect receptor binding; the hinge region of several isotypes carries *O*-glycans; and the variable sequence in the Fab part can contain any number of glycans. To distinguish between glycans at different positions, glycoproteomics methods can be used to generate a site-specific glycosylation profile. Protease-generated glycopeptides can be analyzed using LC-MS/MS, which provides both a relatively high throughput and a good overview of glycan compositions.

In this thesis, mass spectrometry-based glycoproteomics methods were used to characterize the glycosylation of various immunoglobulins. While the field of glycomics is rapidly expanding, antibody glycosylation studies have up to now focused almost solely on IgG, ignoring the other antibody isotypes. This can be partially attributed to the high abundance of IgG in blood and the relatively low complexity of its glycosylation, which simplifies its analysis. Glycosylation of the other isotypes remains largely unstudied, especially for the low-abundant Igs such as IgD and IgE.

Structural Ig glycosylation analysis

Immunoglobulin E exhibits the lowest abundance of all the Igs in blood, but at the same time it has the capacity to ellicit the strongest immune reactions. IgE is involved in protection against parasites, but is mainly known for causing allergic reactions. It's protein sequence contains seven potential *N*-glycosylation sites, but in literature there was no clear consensus as to whether all of these were occupied. In **Chapter 2**, we describe the development of a glycoproteomics method to analyze IgE glycosylation: IgE was digested using three different proteases, and the resulting glycopeptides were identified using LC-MS(/MS). Using this we were able to show that six of the seven potential *N*-glycosylation sites were occupied, and presented a site-specific overview of the *N*-glycans present at each site. We further showed that myeloma-derived IgE exhibited aberrant glycosylation – specifically a higher degree of

tri- and tetra-antennary glycans and a lower degree of bisecting GlcNAc – while IgE from a hyperimmune donor did not, although both these observations were derived from only a single sample. It has since been shown that one of the IgE glycans is essential for binding to the high affinity receptor FcεRI.

In **Chapter 3** we reported partial *O*-glycosylation of IgG3. IgG3 is unique among the IgG subclasses in having an extended hinge region which contains a triple repeat sequence. From LC-MS(/MS) analyses of tryptic glycopeptides, we found that this repeat may contain up to 3 sites of *O*-glycosylation, which carry mainly mono- and di-sialylated core 1-type *O*-glycans. However, only 10% of each site is occupied by a glycan, with little variation in either the occupancy or the type of *O*-glycans seen in six individuals. It can be speculated that the *O*-glycans are involved in protection of IgG3 against bacterial proteases, since they were observed to inhibit digestion with endoproteinase AspN, and because *O*-glycosylation of the IgA1 and IgD hinge has also been shown to confer protease resistance.

Ig glycosylation analysis in population studies

In addition to structural glycosylation research, we also analyzed antibody glycosylation in population cohorts. In **Chapter 4** and **Chapter 5**, IgG Fc glycopeptide analysis was performed on blood samples using LC-MS(/MS). We found that a low degree of galactosylation and sialylation, and a high degree of fucosylation were associated with a state of inflammation.

In a cohort of 76 ANCA vasculitis patients, low galactosylation and sialylation of IgG was associated with a higher chance of future relapse. In relapsing patients, the degree of galactosylation, sialylation and bisection of IgG significantly decreased, while in patients who remained in remission they did not. Interestingly, PR3-specific IgG was not a better predictor of relapse than total IgG, though it reflected the same trends as seen in total IgG.

In the approximately 1800 participants of the Leiden Longevity study (LLS), low galactosylation and sialylation of IgG, together with high fucosylation, showed association with markers of inflammation. Low galactosylation and sialylation also associated with low levels of high-density lipoprotein cholesterol (HDLC) and high levels of tryglycerides, which are known risk factors for cardiovascular disease. Together this points towards a potential role for IgG glycosylation as a biomarker of inflammation and metabolic health. However, the significant inter-individual differences complicate this.

Interestingly, galactosylation and sialylation of IgG2 showed a consistently weaker association with metabolic markers compared to the other IgG subclasses. This could be indicative of a minor role of this IgG subclass *in vivo*, which is supported by *in vitro* receptor affinity studies in literature. Furthermore, in both the LLS and the ANCA vasculitis cohort, we found that after correction for galactosylation, IgG sialylation no longer showed association with metabolic markers or functioned as a predictor for ANCA vasculitis relapse. This supports the theory that mainly galactosylation, and not sialylation, mediates the inflammatory capacity of human IgG. Finally, we found that participants with a latent cytomegalovirus infection exhibited a lower degree of fucosylation, while current smokers exhibited a higher level of bisection.

Conclusion and future prospects

We hope that the novel data presented in this thesis may contribute to the elucidation of the role of antibody glycosylation in the immune system, of which the understanding is currently still very limited. Overall, our data indicates that IgG glycosylation may hold value as a biomarker of inflammation and metabolic health, but the biology behind this proposition is not yet clear. Functional studies have shed some light on the role of IgG glycosylation in receptor binding and other immune reactions, but at the same time this data often clashes with results from *in vivo* associations studies. Therefore, more comprehensive functional studies on IgG glycosylation are warranted, in models which should resemble the human physiology as closely as possible, as well as functional studies on glycosylation of other Igs, which up to now have been scarce.

The sensitivity of current (LC-MS) techniques is rapidly increasing, and we hope that this will enable investigation of Igs such as IgE and IgD, of which the low amount in blood is now a complicating factor for their analysis. Furthermore, in recent years new software tools have been developed to facilitate the analysis of mass spectrometric glycosylation data, which will be of great use for the analysis of antibodies which carry a large number of glycosylation sites, such as IgE and IgM. We therefore expect that high-throughput analysis of glycosylation of all Igs will be within reach shortly.