Immunoglobulin Glycosylation Analysis in Pregnancy and Rheumatoid Arthritis

Analyse van immunoglobuline glycosylering in zwangerschap en reumatoïde artritis

Albert Bondt
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Immunoglobulin Glycosylation Analysis in Pregnancy and Rheumatoid Arthritis

Analyse van immunoglobuline glycosylering in zwangerschap en reumatoïde artritis

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CHAPTER 1

INTRODUCTION

Albert Bondt
Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which occurs in 0.5-1% of the population in industrialized countries. The disease is more prevalent in women than in men, and generally occurs when people are in their forties or older (for women coinciding with menopause), but it also affects many women of childbearing age. RA is diagnosed and classified based upon a set of criteria, including clinical manifestations and laboratory test outcomes (Table 1 and Table 2). Risk factors for developing RA can be genetic, but also smoking and infections are associated with higher prevalence of RA. Characteristic for RA is a symmetrical persistent inflammation of the synovial tissue of hand and feet joints, although all other joints may be involved as well. Furthermore, extra-articular manifestations can be recognized, e.g. fatigue, muscle ache, and cardiovascular complaints. In the affected joints pain and swelling are often observed, and without proper treatment erosions and irreversible destruction may occur.

Pathogenesis

The most established genetic risk factors are involved in T cell activation. Protein tyrosine phosphatase non-receptor type 22 (PTPN22) risk alleles result in stronger inhibition of T and B cell receptor activation, which may result in a less stringent deletion of autoreactive cells. On the other hand, modifications of Human Leukocyte Antigen (HLA)-alleles, in particular the HLA-DRB1 shared epitope, are believed to enhance antigen presentation by antigen-presenting cells leading to increased T cell activation. Antigen presenting dendritic cells are present in inflamed synovium of RA patients. Together with monocytes, macrophages and natural killer cells, which all produce pro-inflammatory cytokines, these cells are able to (co-) stimulate T cells. The pro-inflammatory environment together with genetic predisposition and environmental factors such as smoking show a strong association with the development of autoantibody positive disease, although other variants of the alleles can be protective.

Autoantibodies

In rheumatoid arthritis, being an autoimmune disease, autoantibodies can be found in the majority of patients. These autoantibodies are produced by autoreactive B cells which escaped from the mechanisms in place that should have prevented their development. In the normal situation some autoreactive B cells occur in the circulation, but these are not maturated and not reactive to antigen stimulation. Therefore, no antibody producing autoreactive cells should be present. However, in autoimmune diseases defects in checkpoints generate the presence of fully matured antibody secreting autoreactive plasma cells, producing antibodies of several classes (e.g. immunoglobulin (Ig)M, but also IgG and IgA). It is unclear how the autoantibodies in RA – rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) – exactly are involved in the inflammation of the synovium. Nevertheless it has for example been shown that patients with autoantibodies are less likely to improve during pregnancy, and that these patients generally have a poor
These observations suggest that autoantibody positive and negative RA are different diseases, with their own specific pathogenesis.

Table 1. The 2010 ACR/EULAR classification criteria for RA

<table>
<thead>
<tr>
<th>Classification criteria for RA (score-based algorithm: add score of categories A-D; a score of ≥6/10 is needed for classification of a patient as having definite RA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Joint involvement</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 large joint&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-10 large joints</td>
</tr>
<tr>
<td>1-3 small joints (with or without involvement of large joints)&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-10 small joints (with or without involvement of large joints)</td>
</tr>
<tr>
<td>&gt;10 joints (at least 1 small joint)&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>B. Serology (at least 1 test result is needed for classification)</strong>&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative RF and negative ACPA</td>
</tr>
<tr>
<td>Low-positive RF or low-positive ACPA</td>
</tr>
<tr>
<td>High-positive RF or high-positive ACPA</td>
</tr>
<tr>
<td><strong>C. Acute-phase reactants (at least 1 test result is needed for classification)</strong>&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal CRP and normal ESR</td>
</tr>
<tr>
<td>Abnormal CRP or abnormal ESR</td>
</tr>
<tr>
<td><strong>D. Duration of symptoms</strong>&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;6 weeks</td>
</tr>
<tr>
<td>≥6 weeks</td>
</tr>
</tbody>
</table>

<sup>1</sup>The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfillment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA.

<sup>2</sup>Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

<sup>3</sup>Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

<sup>4</sup>Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement.

<sup>5</sup>“Large joints” refers to shoulders, elbows, hips, knees, and ankles.

<sup>6</sup>“Small joints” refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

<sup>7</sup>In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.).

<sup>8</sup>Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. ACPA = anti-citrullinated protein antibody.

<sup>9</sup>Normal/abnormal is determined by local laboratory standards. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

<sup>10</sup>Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.
1987 American College of Rheumatology (formerly American Rheumatism Association) revised classification criteria for rheumatoid arthritis

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least one hour before maximal improvement.</td>
</tr>
<tr>
<td>Arthritis of three or more joint areas</td>
<td>At least three joint areas (out of 14 possible areas; right or left PIP, MCP, wrist, elbow, knee, ankle, MTP joints) simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) as observed by a physician.</td>
</tr>
<tr>
<td>Arthritis of hand joints</td>
<td>At least one area swollen (as defined above) in a wrist, MCP, or PIP joint.</td>
</tr>
<tr>
<td>Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas (as defined above) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs, without absolute symmetry is acceptable).</td>
</tr>
<tr>
<td>Rheumatoid nodules</td>
<td>Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician.</td>
</tr>
<tr>
<td>Serum rheumatoid factor</td>
<td>Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5 percent of normal control subjects.</td>
</tr>
<tr>
<td>Radiographic changes</td>
<td>Radiographic changes typical of rheumatoid arthritis on posteroanterior hand or wrist radiographs, which must include erosions or unequivocal bony decalcification localised in, or most marked adjacent to, the involved joints (osteoarthritis changes alone do not qualify).</td>
</tr>
</tbody>
</table>

Note: For classification purposes, a patient has RA if at least four of these criteria are satisfied (the first four must have been present for at least six weeks).

Pregnancy

Pregnancy is the only physiological state known to result in spontaneous improvement of RA. During recent years the rates of improvement during pregnancy which been reported have lowered from ±90% to ±50%. This is probably due to a less biased prospective design of more recent studies in comparison to retrospective studies performed earlier. In addition,
due to the increase in treatment options, nowadays more patients enter pregnancy with less active disease and, as a result, show less improvement. The mechanism behind the improvement during pregnancy is unknown, although it is generally believed to be caused by the adaptations of the maternal immune system preventing rejection of the fetus.\textsuperscript{25} This may in part be explained by a shift from a pro-inflammatory Th1 to an anti-inflammatory Th2 subset during pregnancy, but also several other mechanisms have been suggested.\textsuperscript{24,26,27} The pregnancy-associated improvement of RA and the flare of disease activity after delivery suggest the influence of female sex hormones in modulating disease activity.\textsuperscript{23,28} Besides the pregnancy effect the influence of female sex hormones has been suggested because of the higher prevalence of RA (and other autoimmune diseases) in women compared to men, and for example the protective effect of oral contraceptives.\textsuperscript{28} It is furthermore reflected by the high onset of disease in menopause.

An example of a pregnancy hormone associated with modulation of disease activity is prolactin. Levels of this hormone are constitutively increased in RA patients, are associated with disease activity, and may exert their effect by (co-)stimulating macrophages and monocytes to produce pro-inflammatory cytokines.\textsuperscript{29-32} In contrast, estrogen and progesterone have been suggested to possess anti-inflammatory properties.

**RA and pregnancy: the PARA-cohort**

To study the pregnancy effect on RA disease activity in a prospective manner, a large, well described study on RA and pregnancy was designed: the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study. This prospective, nationwide cohort included patients from all over the Netherlands between 2002 and 2010. The patients were visited at their home address pre-conception if possible, three times during pregnancy, and three times after delivery. Information concerning the disease activity and medication use were recorded, and serum and urine samples were collected. More than 250 RA patient pregnancies were included in the cohort. As a reference group data and materials were collected from 32 pregnancies from healthy women at the same time points (with the exception of the pre-pregnancy visit). The goal of the study was to get more insight into the impact of pregnancy on RA disease activity, to study the influence of RA on fertility and pregnancy outcome, and to unravel the mechanisms behind the improvement during pregnancy and the disease activity flare after delivery. This has been and is being done by studying clinical parameters, as well as biochemical and immunological factors.

**Treatment**

Over the last decades treatment of RA has drastically improved by the development of new anti-rheumatic drugs, often therapeutic antibodies targeted against important modulators of inflammation such as TNF, but also by earlier intervention, combination therapy and treat-to-target approaches using methotrexate as the basis for the treatment strategy.\textsuperscript{33} To date, a strong reduction in joint erosion and pain is achieved in most patients by tight
control of a patient’s disease activity, and fast adjustment of medication when targeted goals are not met.\textsuperscript{34}

Treatment during pregnancy is more complicated, because of the known or potential teratogenic effects of the drugs such as methotrexate.\textsuperscript{24,35} However, there is an increasing body of evidence that increased disease activity may also negatively affect pregnancy outcome, and that treatment with selected drugs should be continued.\textsuperscript{24,36,37} Furthermore, high disease activity impairs fertility of the female patients.\textsuperscript{38}

In the period that the PARA-study was performed (2001-2008), physicians were more reluctant of prescribing medication to pregnant women. In that time frame medication was often only prescribed to pregnant arthritis patients with moderate to high disease activity and mainly limited to low dose prednisone, sulfasalazine and to a lesser extent hydroxychloroquine. Due to the very restricted use of medication in the PARA-study, this cohort still offers the opportunity to study the physiological effect of pregnancy on RA-disease activity.

![Figure 1](image)

**Figure 1.** Schematic representation of the common N- (A) and O-glycan (B) structures. N-linked glycans are attached to Asn in a consensus sequence (Asn-Xxx-Ser/Thr, where Xxx \(\neq\) Pro). Three common classes are recognized: complex, high mannose, and hybrid type. For the complex type glycans 4 main characteristics are often described: 1) sialylation, 2) galactosylation, 3) bisection (for ‘bisecting’ N-acetylglucosamine), and 4) fucosylation. O-glycans are linked to Ser/Thr and may consist of various core structures which can be extended. On immunoglobulins Core 1 can be found. Part B is obtained from 39.

**Glycosylation**

A potential immunological aspect on which the pregnancy-associated suppression of the immune system may act is by regulating the downstream effector functions of (auto) antibodies. One suggested mode of action for this phenomenon is by modulating antibody glycosylation.
Antibodies are glycoproteins, which means that they bear a sugar structure connected to the protein backbone. These sugar structures, or glycans, are attached to the protein in a co- and post-translational fashion, in a process called glycosylation. The first precursor glycan is added before protein folding in the endoplasmic reticulum, and it is further processed during the protein maturation in the Golgi apparatus. These glycan moieties may consist of only one up to many building blocks, the monosaccharides. Three distinct classes of N-glycans can be recognized, as depicted in Figure 1A: high-mannose, complex, and hybrid type, for which the latter is a mix of the first two. On antibodies mainly complex type N-glycans with two antennae (diantennary) are found, although some triantennary and high-mannose glycans can also be found. In addition to the N-glycans another type of glycans, namely the O-glycans can be found (Figure 1B). O-glycans are attached to either serine (Ser) or threonine (Thr). The glycans observed on immunoglobulins are generally of the core 1 structure, consisting of an N-acetylgalactosamine (GalNAc), which can be extended by a galactose. In addition, to both the GalNAc and the galactose a sialic acid can be attached. Often multiple O-glycosylation sites are in close proximity of each other, thereby complicating the analysis of such a relatively simple structure. For example on IgA1 up to six occupied O-glycosylation sites can be observed in the hinge region glycopeptide. The majority of the antibody N-glycans found in the circulation carry the monosaccharide sialic acid: sialylation (Figure 1A, characteristic 1). This sugar is believed to control protein clearance via asialo-glycoprotein (glycoproteins without sialic acid) receptors. An exception for the property of being sialylated is IgG, especially its fragment crystallizable (Fc; Figure 2). The IgG-Fc N-glycans appear to be a unique population with high levels of fucose and low levels of galactose and sialic acid. Interestingly, these IgG-Fc N-glycans, in particular the ones without galactose, have been widely recognized for their association with disease activity in RA (Figure 3). Therefore, a large body of literature is available on the (potential) influence of these N-glycans on the IgG effector functions, ascribing both pro- and anti-inflammatory roles to different monosaccharides. (Technically, the terms pro- and anti-inflammatory are not correct, since associations with more or less inflammation is studied and not the action of the individual glycan.) Generally, the effect of the glycan is explained by influencing binding of the IgG to its Fcγ-receptors, or to for example sialic acid binding Ig-like lectins (Siglecs) expressed by immune cells. Many studies have shown associations of overall or protein specific impaired glycosylation with diseases such as cancer, IgA nephropathy, and a variety of autoimmune diseases. In inflammatory diseases such as RA, but also in cancer, high relevance has been ascribed to the previously mentioned N-glycans on IgG-Fc, especially the truncated diantennary complex type glycan, often referred to as G0 or G0F for the lack of galactoses (Figure 3). Not only does the level of galactosylation associate with the disease activity, furthermore the pregnancy-related increase in galactosylation associates with a lowered disease activity. Several studies suggest a role for female sex hormones in the upregulation or adaptation of
enzymes in the glycosylation machinery, which may be a mechanism for immunomodulation in pregnancy and autoimmunity.\textsuperscript{50,51}

Another population of $N$-glycans on IgG has recently gained more attention, namely those on the antigen binding fragment (Fab; Figure 2). These $N$-glycans are present on 15-25% of all IgGs, but show a remarkably high level ($\pm$90%) on ACPA.\textsuperscript{52,53} The role of these glycans has been hypothesized to go via inducing a selection advantage for Fab glycosylated antibody producing B cells, and by prolonging the half-life of the produced ACPAs. This would for example lead to the remarkably high titers of ACPA antibodies.\textsuperscript{53}

Figure 2. Schematic representation of an IgG molecule. The $N$-glycan on the Fc portion is shown at N297, whereas the $N$-glycan on the Fab portion can be anywhere in the variable domain of both heavy and/or light chains as indicated by NXXX.

This figure was originally published in Bondt et al., Immunoglobulin G (IgG) Fab Glycosylation Analysis Using a New Mass Spectrometric High-throughput Profiling Method Reveals Pregnancy-associated Changes. \textit{Mol Cell Proteomics}. 2014; 13:3029-3039. © the American Society for Biochemistry and Molecular Biology, and slightly adapted for the current purpose.
INTRODUCTION

Aims
This thesis aims to provide an increased insight in the role of immunoglobulin glycosylation in rheumatoid arthritis, and how it may play a role in the improvement of disease activity in patients during pregnancy.

The first part of this thesis provides an introduction on the topics covered by this thesis. **Chapter 1** is dedicated to give a concise general introduction to all different topics, whereas **Chapter 2** provides an overview of the state-of-the-art immunoglobulin glycosylation analysis techniques in the year 2016.

In Part II two new methods, developed within the scope of this thesis, are introduced. **Chapter 3** describes a new high-throughput technique to analyze N-glycosylation of the variable domain of IgG at the level of released glycans. **Chapter 4** introduces the development of a new high-throughput method for the analysis of IgA1 glycopeptides.

In **Chapter 5** we applied a recently developed technique for IgG-Fc N-glycosylation analysis on the complete set of samples from over 250 pregnancies collected in the PARA cohort. The most important feature of the new technique was the improved detection of sialic acids, which were unstable in other methods. Thus the application of this method should allow for more detailed information on the glycosylation during pregnancy and after delivery.

For IgG-Fc N-glycosylation the association with RA and pregnancy-associated changes are well described. For IgG-Fab this is not the case, and therefore we applied the method described in Chapter 3 on a subset of the PARA cohort. The results of this are described in **Chapter 6**. In this chapter we studied the differences between RA patients and healthy controls, as well as RA specific phenotypes of Fab glycosylation and its association with disease activity.

Figure 3. Generally IgG N-glycans with galactoses (yellow circle) are associated with low RA disease activity, whereas the lack of galactose is associated with severe disease. Whether or not sialic acids (purple diamond) play a role is still topic of discussion. (We thank Yoann Rombouts for creative input for this figure.)
In addition to IgG antibodies, also antibodies of the IgA class are considered to be of pathogenic significance in RA. Like IgG antibodies, also IgA antibodies bear glycans. In Chapter 7 the method described in Chapter 4 was applied to study disease specific properties of IgA N- and O-glycopeptides, as well as pregnancy-associated modifications thereof. Furthermore, the association with disease activity was explored. Chapter 8 describes the glycosylation analysis of antigen specific IgGs, namely ACPA. Since these autoantibodies are thought to play a role in the pathogenesis of RA, their glycosylation may be more relevant for disease activity, and the pregnancy-associated variation of it. Finally, in Chapter 9 the findings described in this thesis are discussed in a broader context of available literature, and future research is suggested.
References

18. de Man YA, Bakker-Jonges LE, Goorbergh CMD-vd, et al. Women with rheumatoid arthritis negative for anti-cyclic citrullinated peptide and rheumatoid factor are more likely to improve during pregnancy, whereas in autoantibody-positive women autoantibody levels are not influenced by pregnancy. Ann Rheum Dis 2010;69(2):420-23.


CHAPTER 2

RECENT ADVANCES IN CLINICAL GLYCOPROTEOMICS OF IMMUNOGLOBULINS

Rosina Plomp, Albert Bondt, Noortje de Haan, Yoann Rombouts, Manfred Wuhrer

Abstract

Antibody glycosylation analysis has seen methodological progress resulting in new findings with regard to antibody glycan structure and function in recent years. For example, antigen-specific IgG glycosylation analysis is now applicable for clinical samples due to the increased sensitivity of measurements, and this has led to new insights in the relationship between IgG glycosylation and various diseases. Furthermore, many new methods have been developed for the purification and analysis of IgG Fc glycopeptides, notably multiple reaction monitoring for high-throughput quantitative glycosylation analysis. In addition, new protocols for IgG Fab glycosylation analysis were established revealing autoimmune disease-associated changes. Functional analysis has shown that glycosylation of IgA and IgE is involved in transport across the intestinal epithelium and receptor binding, respectively.
Introduction
Glycosylation of immunoglobulins (Igs) plays a key role in the regulation of immune reactions: glycans located at various sites modulate a diversity of immunoglobulin properties including protein conformation and stability, serum half-life, as well as binding affinities to antigens, receptors and glycan-binding proteins (GBP).\(^1\)\(^-\)\(^3\) The five classes of human antibodies – IgG, IgA, IgM, IgE and IgD – each contain one to six sites for \(N\)-linked glycosylation within the conserved sequence of each heavy chain\(^4\). IgA1, IgD and IgG3 also carry \(O\)-linked glycans on their hinge-region.\(^4\),\(^5\) In addition, immunoglobulins can be glycosylated in the variable domain of the Fab (antigen-binding fragment).\(^5\)\(^-\)\(^8\) Importantly, glycosylation adds a formidable degree of complexity to protein species, since a range of glycan structures is usually present at each glycosylation site.

Studies on the functional consequences of immunoglobulin glycosylation, especially for IgG, have shown that glycans linked to the Fc (fragment crystallizable) part of the antibody influence the interaction with Fc receptors and GBPs, thereby regulating the pro- or anti-inflammatory immune response.\(^1\),\(^9\)\(^-\)\(^12\) For example, lack of a fucose on the IgG Fc glycan can enact a 100-fold increase in antibody-dependent cellular cytotoxicity (ADCC).\(^13\),\(^14\) Fc-linked glycans may also influence the endocytosis, transcytosis and half-life of some classes of immunoglobulin, such as IgA.\(^15\),\(^16\) Next to Fc-linked glycosylation, glycans attached to the Fab region also influence Ig properties and inflammation, especially by modulating antigen recognition and antibody aggregation, as well as through the binding to GBP.\(^7\),\(^17\) Importantly, antibody glycosylation has been shown to reflect the physiological and pathological condition of an organism.\(^18\)\(^-\)\(^20\)

Because of the impact on the immunological response and thus the efficacy of therapeutic antibody treatment, it is crucial to monitor and in some cases alter the glycosylation profile in order to optimize antibody effector functions.\(^9\),\(^13\) Glycosylation of antibodies can vary widely depending on the expression system and cell culture conditions during production.\(^13\) Because non-human glycan structures can trigger immunogenic responses, therapeutic antibodies are currently produced exclusively in mammalian cell cultures. Due to improvements in glyco-engineering, it is expected that non-mammalian expression systems will soon be applicable as well.\(^13\),\(^21\) Robust and high-throughput methods are needed to monitor the glycosylation of therapeutic antibodies. Additionally, glycosylation analysis should be site-specific since the function of a glycan can depend on its location, as illustrated by the different influence of glycans located at the Fc and at the Fab part of IgG.\(^12\),\(^17\)

Glycosylation profiling of antibodies is usually done using one of the following approaches: 1) by releasing glycans from the protein, which is easily done for \(N\)-glycans by digestion with PNGase F, while \(O\)-glycans can be released chemically through hydrazinolysis or beta-elimination; 2) by using a proteolytic enzyme to digest the glycoprotein, resulting in glycopeptides; or 3) by analyzing the intact glycoprotein or portions thereof (e.g. Ig heavy and light chains).\(^22\)\(^-\)\(^24\) Recent years have seen major methodological advances in all three
approaches as detailed in this review. In addition, selected examples are given of antibody glycosylation studies in both biotechnological and biomedical research.

Table 1. Several different immunoglobulin protein sequence nomenclatures are used in literature. The nomenclature most frequently used in literature is based on archaic sequencing data of both immunoglobulin variable and constant domains, whereas the UniProt numbering is based on the conserved sequences, and the IMGT nomenclature is based on homology between the immunoglobulins.

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>conventional literature</th>
<th>UniProt</th>
<th>IMGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>297</td>
<td>180</td>
<td>CH2-84.4</td>
</tr>
<tr>
<td>IgG2</td>
<td>297</td>
<td>176</td>
<td>CH2-84.4</td>
</tr>
<tr>
<td>IgG3</td>
<td>297</td>
<td>227</td>
<td>CH2-84.4</td>
</tr>
<tr>
<td>IgG3</td>
<td>392</td>
<td>322</td>
<td>CH3-79</td>
</tr>
<tr>
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</tr>
<tr>
<td>IgM</td>
<td>171</td>
<td>46</td>
<td>CH1-45</td>
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<tr>
<td>IgM</td>
<td>332</td>
<td>209</td>
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</tr>
<tr>
<td>IgM</td>
<td>395</td>
<td>272</td>
<td>CH3-81</td>
</tr>
<tr>
<td>IgM</td>
<td>402</td>
<td>279</td>
<td>CH3-84.4</td>
</tr>
<tr>
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<td>563</td>
<td>439</td>
<td>CH5-7</td>
</tr>
<tr>
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<td>140/145</td>
<td>21</td>
<td>CH1-15.2</td>
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<tr>
<td>IgE</td>
<td>168/173</td>
<td>49</td>
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</tr>
<tr>
<td>IgE</td>
<td>218/219</td>
<td>99</td>
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<tr>
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<td>265</td>
<td>146</td>
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</tr>
<tr>
<td>IgD</td>
<td>496</td>
<td>367</td>
<td>CH3-116</td>
</tr>
</tbody>
</table>

1 As used in e.g. (4)
2 (26)
3 (25)
4 alternative nomenclature used in (121)
In the field of immunoglobulin (glyco)proteomics, several nomenclatures for the glycosylation sites are used (Table 1). The one most commonly used refers to the Asn positions as determined in the old days based on Edman sequencing of both variable and heavy chains (e.g.\(^4\)) Alternatively, the homology-based nomenclature by the international ImMunoGeneTics information system (IMGT) is available for immunoglobulins, which has the advantage of a more intuitive comparison between the different immunoglobulins (e.g. site homology between CH2 84.4 on IgG and IgD, as well as similarity with CH3 84.4 on IgE and IgM). \(^{25}\) In this review we will use the UniProt based site annotation, since this is more easily integrated with proteomic databases. \(^{26}\)

Analysis of the antibodies themselves is complicated by the variable domain which dictates the specificity of the antigen-binding site. Protein sequencing of monoclonal antibodies or affinity-purified antibodies is done using high resolution liquid chromatography tandem mass spectrometry methods, coupled to DNA sequence information generated by next-generation sequencing of the B-cell antibody repertoire. \(^{27, 28}\)

Post-translational modifications, such as glycosylation, further complicate antibody analysis and require specific analysis strategies, as will be detailed in this review.

**IgG**

**Fc glycosylation at Asn180/176/227/177 (‘Asn297’)**

The majority of IgG glycosylation analysis has been focused on the Fc glycan because of both the known influence of this glycan on IgG effector functions and the established high-throughput methods which are available to selectively monitor this glycosylation site. \(^{12, 23, 24}\) In human IgG, the conserved \(N\)-glycosylation site is located at Asn180 (IgG1; UniProt P01857), Asn176 (IgG2; P01859), Asn227 (IgG3; P01860) or Asn177 (IgG4; P01861), alternatively referred to as position CH2-84.4 \(^{25}\) or Asn297 (e.g. in\(^4\); Table 1). In all IgG subclasses, the Fc-glycosylation site has been shown to harbor complex type diantennary \(N\)-glycans which carry between zero and two galactoses, with the majority carrying a core fucose, and a minority having a bisecting \(N\)-acytlyglucosamine (GlcNAc) and one or two sialic acids. \(^{29}\) The glycan at this site has been shown to influence the inflammatory capacity of IgG through modulation of the binding to Fc-gamma receptors (Fc\(\gamma\)Rs) and C-type lectins: in general, the absence of a core fucose and/or absence of galactoses and sialic acids appear to convey pro-inflammatory properties, while the presence of terminal sialic acids triggers an anti-inflammatory response. \(^{1, 12, 30}\)

Changes in Fc glycosylation, i.e. a decrease in galactosylation and sialylation which contributes to a more inflammatory antibody profile, have been observed in various autoimmune disorders, most recently inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and chronic inflammatory demyelinating polyneuropathy (CIDP). \(^{31-34}\) In addition to autoimmune disorders, IgG glycosylation changes can also occur in infectious diseases, as was shown by recent studies on HIV infection, chronic hepatitis B and the parasitic disease visceral leishmaniasis. \(^{35-37}\) Furthermore, new reports reaffirm
the potential role for IgG glycosylation as a biomarker for cancer progression.\textsuperscript{38, 39} Finally, congenital defects in glycosylation or carbohydrate metabolism also alter IgG glycosylation, as shown recently for Man1B1 deficiency and galactosemia.\textsuperscript{40, 41}

**Analysis of released glycans**

The gold standard for studying IgG glycosylation relies on enzymatic $N$-glycan release, subsequent fluorescent labeling by reductive amination and analysis of the labeled glycans by high-performance liquid chromatography (HPLC) using hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection.\textsuperscript{42} First, this approach has been further developed by implementing HILIC stationary phases for ultra-performance liquid chromatography (UPLC) instrumentation, thereby improving both throughput and resolution.\textsuperscript{43} Second, sample preparation has been simplified through the use of 96-well filter plates to increase the throughput of glycan purification,\textsuperscript{44} as well as by introducing fluorescent tags to label the glycosylamine species released by PNGase F, instead of targeting the aldehyde species that arise from acid-catalyzed hydrolysis of the glycosylamine.\textsuperscript{45} Of note, the increased throughput capacity allowed for analyses of large sample sets, which could for example show the potential of the IgG $N$-glycans as a marker of chronological and biological age.\textsuperscript{46} Third, sample preparation has been robotized, resulting in a highly automated, higher-throughput workflow and leading to more robust results.\textsuperscript{45} However, this method has a disadvantage: because the glycans are released from the IgG, it is impossible to distinguish between Fab and Fc glycans as well as between glycans originating from different IgG subclasses.

Next to HILIC UPLC of fluorescently labeled glycans, various methods for repetitive IgG glycosylation analysis (“profiling”) have reached maturity as evidenced by the high consistency of the results obtained in extensive method comparison studies.\textsuperscript{22-24} Remarkably, various mass spectrometric methods showed very good performance with respect to resolution, sensitivity and robustness, which opened the way to their broad application in both biotechnological\textsuperscript{23, 24} and biomedical applications.\textsuperscript{22}

**Analysis of glycopeptides**

A bottom-up proteomics approach, with trypsin digestion followed by liquid chromatography (LC) coupled to mass spectrometric analysis, is most commonly applied for site-specific analysis of IgG Fc glycosylation.\textsuperscript{23} Tryptic digestion results in distinct glycopeptides that allow discrimination of the different IgG subclasses – with the peptide moieties EEQYNSTYR for IgG1, EEQFNSTFR for IgG2 and EEQFNSTYR for IgG4. The peptide sequence of the IgG3 glycopeptide shows allotype variation in the amino acid at the position $N$-terminal of the Asn227, causing a mass that is identical to either the IgG2 peptide (EEQFNSTFR; predominant allotype in Caucasian populations) or the IgG4 sequence (EEQYNSTFR; predominant allotype in Asian and African populations).\textsuperscript{47} While trypsin digestion forms the gold standard for
Fc IgG glycopeptide analysis, we have recently found that incomplete denaturation and digestion of IgG might lead to biases in glycoprofiling.\textsuperscript{48} To prevent ion suppression of the glycopeptides by unglycosylated peptides during mass spectrometric analysis, a glycopeptide separation or enrichment step is often applied. This separation is usually performed using either reverse phase LC with C18 or graphitized carbon as a stationary phase, or by HILIC.\textsuperscript{2} While a variety of stationary phases exist for HILIC, amide is most frequently used, although polysaccharide-based stationary phases may show similar performance. Notably, the affinity of current HILIC materials is often dependent on glycan structure, which can lead to a bias in enrichment.\textsuperscript{2} The lack of a gold standard method has led to the development of various new HILIC materials for glycopeptide enrichment of tryptic IgG (glyco)peptides, with stationary phases consisting of various polysaccharides (chitosan, dextran, cyclodextrin, maltose) coupled to magnetic particles, silica particles or metallo-organic frameworks,\textsuperscript{49-52} or functionalized amide polymers embedded in a monolith capillary.\textsuperscript{53} Furthermore, electrostatic repulsion HILIC (also known as ERLIC) has been successfully employed to enrich IgG glycopeptides, although a thorough analysis of the potential skewing of IgG glycoforms in ERLIC enrichment is still lacking.\textsuperscript{54} Zwitterionic (ZIC) HILIC has also gained popularity: this technique makes use of highly hydrophilic materials carrying both positive and negative charges and shows a very good performance in glycopeptide enrichment.\textsuperscript{55} Novel ZIC HILIC materials, consisting of zwitterionic polymers coupled to silica particles or magnetic nanoparticles, have been developed and feature high sensitivity in post-enrichment MALDI-MS measurements.\textsuperscript{56-58} Multiple reaction monitoring (MRM) is well-suited for high-throughput quantitative analysis of complex samples and has only recently been applied to glycopeptide Ig analysis.\textsuperscript{59-61} Hong et al. developed a method which simultaneously performs glycoprofiling of IgG and absolute quantitation of IgG and its separate subclasses, which can help determine if a relative change in glycosylation is due to changes in post-translational modification or changes in the level of protein production.\textsuperscript{61} For the quantification of glycopeptides, MRM was set to specifically detect oxonium ions, fragment ions that originate during the fragmentation of glycopeptides. Similar methods are being developed for IgA and IgM.\textsuperscript{59} A comparable protocol describing MRM detection of IgG glycoforms was separately developed by Yuan et al., with the added feature of a prior separation of IgG3 so that glycosylation could be observed separately for each of the four IgG subclasses.\textsuperscript{62} Capillary electrophoresis (CE) coupled to MS has likewise been applied for tryptic IgG analysis and shows a vastly increased sensitivity compared to the LC-MS approach.\textsuperscript{63} The sensitivity gain may be largely ascribed to the very low flow rates achieved in CE-MS. Compared to LC-ESI-MS or CE-MS, MALDI-MS offers higher sample throughput as well as lower data complexity. However, detection of glycopeptides is complicated by the loss of sialic acids through in-source decay. Of note, this can be prevented by neutralizing the charge on the sialic acid by (dimethyl-)amidation\textsuperscript{29, 64} or ethyl esterification.\textsuperscript{65} The derivatization methods target carboxyl groups on the peptide moiety as well as on the glycan, which can
provide useful structural information from a combination of positive- and negative-ion MS/MS analyses. An additional advantage of these methods is the introduced mass difference between α2,3- and α2,6-linked sialic acids, caused by the sialic acid linkage-specificity of the reactions. While the ethyl esterification method is highly specific for sialic acid linkages on the level of released glycans, the modification of the peptide moiety of glycopeptides was found to be not completely specific, resulting in unwanted byproducts. This issue has been addressed and overcome by a recently published method using dimethylamidation, which provides sialic acid linkage information on a stably modified glycopeptide (Figure 1).

Derivatization results in the stabilization of the sialylated glycopeptides, improving their detection and preventing the formation of metastable signals. With the use of dimethylamidation, no unspecific side reactivity on the peptides was observed and the linkage of the sialic acids could be determined (29).

Figure 1. MALDI-TOF-MS spectra of human plasma IgG Fc-glycopeptides (A) without derivatization, and (B) after dimethylamidation.

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by MALDI-MS and also allows discrimination between α2,3- and α2,6-linked sialic acids, although its application on immunoglobulins has not been established.

Furthermore, stable isotope labeling of glycopeptides has recently been achieved: succinic anhydride was used to introduce a mass increment of 8 Da to tryptic IgG1 glycopeptides. This method can be used to perform absolute quantification of IgG glycopeptides, or can be used to reduce bias during sample analysis by parallel analysis of two samples. Isobaric labeling using tandem mass tags (TMTs), which lead to different masses upon fragmentation, has also been used for the analysis of pepsin-generated IgG glycopeptides.

**Analysis of intact glycoprotein or glycoprotein fragments**

Alternatively, a middle-down or middle-up proteomics approach can be applied for assessing IgG Fc glycosylation. The Fc portion can be cleaved from the Fab portion in whole IgG, or from fused proteins in Fc-fusion proteins, by limited proteolytic digestion with the protease IdeS (FabRICATOR®), followed by either mass spectrometric analysis of the protein fragment or purification of the Fc portion and release of the glycans using PNGase F. The latter method has recently been applied to a clinical sample set, as is discussed later in this review.

Finally, technical advances in the recent years have allowed for top-down mass spectrometric analysis of monoclonal antibodies, allowing the integrated analysis of post-translational modifications. An extensive review has been recently published by Zhang et al., describing several applications of these techniques, and a comparison between ‘normal’ and native MS, furthermore including ion mobility. More recently, ultra-high resolution machinery (e.g. Fourier transform ion cyclotron resonance (FTICR)) allowed for the detection of intact monoclonal antibodies with isotopic resolution, showing several glycoforms. Additional top-down MS/MS information was obtained in conjunction with online electrochemical reduction of the antibody. Furthermore, isobaric labeling has been applied to intact antibody-drug conjugates. Native MS is often applied to analyze intact mAbs. The advantage of this approach is the limitation of charge states because of the native 3D protein configuration, causing increased signal-to-noise for the few charge states that do occur. The downside of native MS is a lack of information regarding the glycosylation site(s) or the precise structure of the glycan(s).

**Fab glycosylation**

The structural features of IgG Fab glycosylation and its emerging importance in immunity have been recently reviewed. It has been estimated that approximately 15-25% of serum IgG of healthy individuals contain N-glycosylation sites and carry N-glycans (Fab-glycosylation) in their variable domains, in addition to the almost fully occupied IgG Fc N-glycosylation site. Of note, the percentage of Fab-glycosylation and glycan structures varies during certain pathological and physiological conditions, as shown in RA, lymphoma and pregnancy. Since only a few germline-encoded sequences contain an N-glycosylation site, the sites present in the variable domains of immunoglobulins are mainly introduced by somatic
hypermutation during the process of affinity maturation.\textsuperscript{8, 82} Within an affinity-purified population of antigen-specific IgG, identification of Fab glycosylation sites has been achieved by labeling the sites with \textsuperscript{18}O during deglycosylation with PNGase F. This was followed by mass spectrometry-assisted proteomics analysis which revealed the mass shift denoting the site of glycosylation and the peptide sequence surrounding the site.\textsuperscript{7} The human immune system comprises of an enormous antibody repertoire, recognizing an estimated billion or more different antigens. Antibody specificity is determined by a unique amino acid sequence in the Fab portion, thus making the analysis of Fab \textit{N}-glycopeptides derived from polyclonal antibodies very difficult, if feasible at all.

Therefore, in order to analyze polyclonal IgG Fab glycosylation, the currently used analytical methods consist of the release of \textit{N}-glycans from purified Fab fragments followed by their analysis using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), (ultra)high performance liquid chromatography and/or mass spectrometry.\textsuperscript{6, 7, 83} In order to improve the throughput of IgG Fab glycosylation analysis, we recently set up a new sample preparation method. The method relies on IgG affinity capturing in a 96-well filter plate, on-bead proteolytic release of the Fab portions, and collection of Fab (flow-through) and Fc portions (eluate) followed by enzymatic glycan release. Detailed glycan information was obtained by MALDI-TOF-MS after sialic acid stabilization. The method was applied to study the differences between Fab and Fc glycosylation in young women, and the pregnancy associated changes thereof.\textsuperscript{6} The levels of galactosylation, sialylation and bisection were significantly higher on the Fab portion compared to the Fc. During pregnancy Fab and Fc glycans showed similar patterns in their changes. Interestingly, the Fab portion was also shown to carry minor amounts of α2,3-linked sialic acids. In general, the \textit{N}-glycans on the Fab are more extended, and some species seem to be Fab-specific. Diantennary fucosylated glycans with two sialic acids are hardly present on the Fc, while they are the major species on Fab. Similarly, the presence of a bisecting GlcNAc on glycans with two galactoses appears to be more prominent in Fab versus Fc.\textsuperscript{6} Of note, these data result from the analysis of solely young women, while characterization of Fab glycosylation in males as well as different age groups is still lacking.

High-sensitivity analysis of released Fab glycans can also be performed by CGE-LIF.\textsuperscript{83} However, the glycosylation data obtained via these techniques, especially regarding both the levels of \textit{N}-glycan bisection in IgG Fab portions and various glycosylation features of murine IgG, has shown some discrepancies as compared to results obtained with other analytical methods.\textsuperscript{6, 84, 85} Additional studies are needed to further unravel Fab glycosylation changes with age, sex and diseases.

**Additional \textit{N}- and \textit{O}-glycosylation of IgG3**

In addition to the well-known Fc \textit{N}-glycosylation site, several allotypes of IgG3 possess a second \textit{N}-linked site in the CH3 domain at Asn322 (UniProt P01860; alternatively referred to as CH3-79\textsuperscript{25} or Asn392; Table 1).\textsuperscript{86} Only 10% of Asn322 was found to be occupied; the
N-glycans found at this site were mainly complex type diantennary structures, which differ from those at Asn227 in that the majority is afucosylated and contains a bisecting GlcNAc, and a minority of high mannose type N-glycans is also present.\textsuperscript{86} Since trypsin digestion produces a very large glycopeptide containing this site, the glycan structures may alternatively be examined after digestion with aspecific proteases. Aspecific proteases such as pronase are particularly useful for the study of glycoproteins since they tend to produce small glycopeptides which are well suited for mass spectrometric analysis.\textsuperscript{87, 88} Sequential chromatography of resulting digests on a C18 reversed phase column and a porous graphitized carbon column provides the broad retention range necessary to observe all glycopeptides regardless of the heterogeneous retention properties of both the glycan and the peptide moiety. Using collision-induced dissociation with a combination of lower- and enhanced-energy, which produced both glycan and peptide fragmentation, respectively, identification of both the glycan and the peptide moiety in one run could be achieved.\textsuperscript{86} Next to N-glycosylation, IgG3 may carry up to 3 O-glycosylation sites per heavy chain within a triple repeat in the hinge region.\textsuperscript{5} Proteolytic digestion with trypsin, proteinase K or pronase followed by LC-MS/MS analysis revealed that approximately 10% of each of these sites is occupied, mainly by sialylated core 1 type O-glycan structures.\textsuperscript{5, 86} As both the Fc N-glycosylation at Asn322 and the O-glycans of IgG3 have been described only recently, their function and clinical relevance remain to be investigated.

\textbf{Antigen-specific IgG}

The glycoprofiling of antigen-specific antibodies in clinical samples after vaccination or during disease started less than a decade ago. This was made possible by the numerous improvements in sensitivity and throughput of methods for both antibody purification and glycosylation analysis. Antigen-specific antibodies are generally purified by affinity chromatography using antigens coated on 96-well plates or on chromatography beads/columns. Antigens are usually synthetic peptides or recombinant (glyco)proteins. For instance, the high-throughput purification of anti-citrullinated peptide/protein antibodies (ACPA), i.e. autoantibodies specific for rheumatoid arthritis (RA), has been achieved by repeated capturing on 96-well plates coated with a synthetic circular peptide containing citrulline, called CCP2 (cyclic citrullinated peptide 2).\textsuperscript{19, 89, 90} Likewise, antibodies directed against multiple HIV and influenza antigens (e.g. HIV gp41 and gp120 or influenza hemagglutinin) have been enriched using amino-link antigen resin or antigen-functionalized streptavidin resins packed into cartridges.\textsuperscript{36, 91, 92} Alternatively, antigen-specific antibodies can be captured by using viral particles, microorganisms or cells. Thus, Vidarsson and coworkers have isolated anti-platelet antibodies (causing neonatal alloimmune thrombocytopenia) and anti-red blood cell antibodies (responsible for haemolytic disease of the fetus and newborn) by incubating serum of pregnant women directly on platelets and red blood cells.\textsuperscript{93-95} Fc- and/or Fab-linked glycosylation of antigen-specific IgG has been analyzed either at the glycopeptide level mainly using LC-MS, or by releasing glycans using a middle-down/middle-
up proteomics approach as described above. Of note, unlike glycopeptide detection, analysis of released glycans from antigen-specific IgG requires another purification step prior to or after antigen-specific capturing in order to separate IgG from other serum glycoproteins or other immunoglobulins.\textsuperscript{91}

Antigen-specific IgG displays different sialylation, galactosylation, fucosylation and/or bisection patterns compared to total IgG isolated from the same individuals. Importantly, these structural differences are clearly associated with clinical and functional consequences including disease outcome, disease severity and/or antiviral control responses. For instance, as compared to total IgG, anti-platelet IgGs found in the serum of pregnant women exhibit an exceptionally low level of fucosylation in their Fc-glycans, which enhances the binding affinity for the FcyRIIIa/b and the phagocytosis of platelets, and correlates with increased severity of neonatal alloimmune thrombocytopenia.\textsuperscript{95} Likewise, HIV-specific IgG antibodies isolated from HIV-positive subjects present a higher frequency of afucosylated, agalactosylated, and asialylated N-glycans compared to total IgG. Importantly, this glycan difference, especially the greater percentage of agalactosylated glycoforms, is far more pronounced in HIV elite controllers than in (un)treated chronic progressors and is associated with an enhanced capacity to bind to FcyRIIIa, probably explaining the more potent antibody-dependent cellular viral inhibition activity that characterizes antibody from elite controllers.\textsuperscript{96} A disruption in the balance between type I (part of the Ig receptor superfamily which includes FcyRI, II and III) and type II (C-type lectin receptors) Fc receptor signaling also very likely occurs in several autoimmune diseases such as rheumatoid arthritis and granulomatosis with polyangiitis (GPA), in which changes in autoantibody-specific glycosylation have been observed. Thus, the Fc-galactosylation, sialylation, and bisection of anti-proteinase 3 (PR3) antibodies IgG1 are reduced compared to total IgG1 in GPA patients.\textsuperscript{96} Despite an early study reporting a negative correlation between the level of anti-PR3 specific IgG sialylation and disease activity as measured by the Birmingham Vasculitis Activity Score (BVAS), recent evidence demonstrated that the BVAS is strongly associated with the presence of bisecting GlcNAc on anti-PR3 IgG but not with galactosylation/sialylation percentages.\textsuperscript{96, 97} Interestingly, the level of anti-PR3 IgG galactosylation was associated with pro-inflammatory cytokine concentrations and time to remission.\textsuperscript{96} Similarly, in RA patients, ACPA-IgG autoantibodies exhibit a decrease in Fc galactosylation and sialylation levels that occurs a few months before disease presentation, correlates with disease severity, and potentially determines osteoclast differentiation and bone loss during RA.\textsuperscript{19, 89, 98} Variations in antigen-specific IgG glycosylation have also been observed following vaccination and, more importantly, can predict the efficacy of vaccination.\textsuperscript{92, 99} Ravetch and coworkers recently showed that the sialylated Fc glycan abundance on anti-hemagglutinin IgG produced by day 7 following influenza virus vaccination predicts the quality of the vaccine response.\textsuperscript{92} It was proposed that immune complexes formed with Fc-sialylated IgG signal through the type II FcR CD23 on activated B cells and triggers the expression of FcyRIIb, thereby driving the selection of higher affinity B cells and the generation of higher affinity and more protective anti-HA IgG.\textsuperscript{92}
IgA

There are two subclasses of immunoglobulin A (i.e. IgA1 and IgA2), and two known allotypes for the IgA2 subclass (i.e. A2m(1) and A2m(2)). IgA1 contains a slightly elongated hinge region compared to IgA2. This elongated hinge contains 9 potential O-glycosylation sites, of which up to six have been reported to be occupied. In addition, IgA1 harbors two N-glycosylation sites at Asn144 and Asn340 (UniProt P01876; alternatively referred to as CH2-20 and CHS-7, respectively, or Asn263 and Asn459 (e.g. in4); Table 1), whereas IgA2 harbors four sites at Asn47, Asn131, Asn205, and Asn327 (UniProt P01877; also referred to as CH1-45.2, CH2-20, CH2-120, CHS-7, or Asn166, Asn263, Asn337, and Asn459 (e.g. in4); Table 1). In the A2m(2) allotype of IgA2 an additional consensus sequence is present due to the replacement of a proline by a serine, thus forming a glycosylation site at Asn92 (CH1-114 / Asn211; Table 1). The analysis of (s)IgA glycosylation is generally performed at the level of released glycans or by lectin ELISA, although a few glycopeptide-based LC-MS/MS methods have been described.

For the analysis of tryptic O-glycopeptides, the use of FT-MS/MS coupled online to an RP-LC system has been described. Electron transfer dissociation, which preferentially fragments the peptide backbone and not the glycan, was used to determine the location of each glycosylation site. By applying a few additional separation steps using less advanced laboratory techniques, others have achieved similar results by MALDI-TOF/TOF-MS.

The IgA N-glycosylation is less frequently studied, although it may have important functional consequences as demonstrated by the influence of N-glycan sialylation on the transportation of secretory IgA across an in vitro model of follicle-associated epithelium via binding to Dectin-1 and Siglec-5.

Nowadays, the use of IgA instead of IgG monoclonal antibodies for biopharmaceutical purposes is being explored, with a focus on anti-HIV drugs. Therefore, several site-specific glycosylation analysis methods have recently been developed. An LC-ESI-MS/MS method primarily developed for the analysis of HIV gp140 has been adapted and applied to secretory IgA1 produced in plants as well as to human IgA. In brief, IgA was digested by sequential application of trypsin and GluC after reduction and alkylation. Next, glycopeptide analysis was performed by first identifying the elution position of deglycosylated peptide moieties. Glycopeptides are known to elute a short time ahead of the deglycosylated variant, with some spread due to the various glycans attached. The addition of a buffered formic acid solution to the flow ascertained very close or even identical elution times for glycosylated peptides bearing sialylated structures. A targeted search for the peptide plus potential glycan m/z using selected ion chromatograms completed the analysis. Several high mannose type structures were identified using the applied technique. The analysis did not reveal any hinge region O-glycosylation, which could be attributed to the production in plants.

The N-glycans of IgA are nevertheless still mainly studied at the level of released glycans. For example, the comparison of released glycans from different IgA constructs obtained from
various cell lines showed profound differences, especially regarding the level of sialylation, which correlated with the half-life of these antibodies.\textsuperscript{111} Of note, the secreted form of IgA consists of a dimer, which forms a complex with the (also glycosylated) joining (J)-chain and the secretory component. The J-chain harbors a single glycosylation site at Asn71 (UniProt P01591; also referred to as Asn48), which appears to be important for IgA dimerization.\textsuperscript{112} This site bears mainly highly sialylated diantennary N-glycans.\textsuperscript{101, 104} The secretory component is also highly glycosylated, with seven N-glycosylation sites at Asn83, Asn90, Asn135, Asn186, Asn421, Asn469 and Asn499 (UniProt P01833; also referred to as Asn65, Asn72, Asn117, Asn168, Asn403, Asn451, and Asn481, e.g. in\textsuperscript{113}; Table 1). The protein contains a wide variety of glycan species: di-, tri- and tetraantennary glycans, bearing all Lewis epitopes.\textsuperscript{101, 104} It was suggested that these glycans are meant to bind to lectins of bacteria.\textsuperscript{101}

\textbf{IgM}

Human serum IgM mainly circulates as a pentamer of 950 kDa consisting of ten light chains, ten heavy chains and one joining chain (J-chain). Each IgM monomer contains five conserved N-glycosylation sites at Asn46, Asn209, Asn272, Asn279 and Asn439 (UniProt P01871; also known as CH1-45, CH2-120, CH3-81, CH3-84.4, CHS-7,\textsuperscript{25} or Asn171, Asn332, Asn395, Asn402, and Asn563; Table 1) located within the constant region of the heavy chain. In addition, the previously mentioned J-chain contains one N-glycosylation site at Asn71 (UniProt P01591). In two recent studies, a site-specific N-glycosylation mapping of human serum IgM was achieved by analyzing IgM glycopeptides, generated by trypsin or trypsin/GluC digestion, using either the classical LC-ESI-MS method or a nano-LC-microarray-MALDI-MS platform.\textsuperscript{114, 115} The latter consists of a nano-LC reverse phase separation of IgM (glyco) peptides, including the J-chain glycopeptide, followed by high frequency droplet-based fractionation of the nano-LC outflow on microarray chips. Each spot on the microarray is then analyzed by MALDI-MS, with or without pre-digestion with PNGase F to remove N-glycans. Both studies demonstrated that glycans linked to Asn279 and Asn439 are predominantly oligomannose structures, whereas glycans attached to Asn46, Asn209 and Asn272 mainly consist of complex-type structures.\textsuperscript{114, 115} The glycosylation site Asn71 of the J-chain also exhibits complex-type N-glycans. The main complex-type N-glycans found in IgM heavy chains are diantennary species carrying one or two sialic acids, bisecting GlcNAc and/or a core fucose. Minor proportions of oligomannosidic and hybrid-type glycans were also detected on Asn46.\textsuperscript{114} Likewise, Asn279 carries 10% of hybrid-type structures, which are also present in very low amount on Asn209. Based on computer modeling of the IgM structure, the clear distinction between glycosylation sites carrying oligomannose structures (on Asn279 and Asn439) or complex-type N-glycans (on Asn46, Asn209, Asn272) has been proposed to be the consequence of the low accessibility of glycans on Asn279 and Asn439 for the glycosyltransferase/glycosidases within the Golgi.\textsuperscript{115} Finally, although the functional aspect of IgM glycosylation on immunity has not been examined yet, the recent possibility of
producing human-like glycoengineered heteromultimeric IgM in plants may help to provide new insights in this field.\textsuperscript{115}

**IgE**

With six oligosaccharides on each heavy chain at Asn21, Asn49, Asn99, Asn146, Asn252 and Asn275\textsuperscript{116} (UniProt P01854; also referred to as CH1-15.2, CH1-45.2, CH1-118, CH2-38, CH3-38 and CH3-84.4\textsuperscript{25}, or Asn140, Asn168, Asn218, Asn265, Asn371 and Asn394 e.g. in\textsuperscript{4}; Table 1), IgE is the most heavily glycosylated of the immunoglobulins. Characterization of the glycan structures on polyclonal IgE was achieved with a combination of proteolytic enzymes and LC-MS/MS analysis.\textsuperscript{116} Glycosylation sites Asn21, Asn49, Asn99, Asn146 and Asn252 are occupied by complex type N-glycans, which are primarily fully galactosylated diantennary structures, containing a core fucose and one or two sialic acids.\textsuperscript{116, 117} A high mannose type glycan is present at Asn275, the sixth site, which is homologous to the Fc glycosylation site in IgG. Glycosylation at this site has recently been shown to be essential for the binding of IgE to the high affinity receptor FcεRI and initiation of anaphylaxis.\textsuperscript{118} Individuals with PGM3-related hyper IgE syndrome or with a hyperimmune condition displayed similar IgE glycosylation compared to healthy individuals.\textsuperscript{116, 117} Glycosylation analysis of IgE is challenging due to the low concentration in biological fluids: at approximately 130-300 ng/mL, the concentration in human serum is roughly 50 000 times lower than that of IgG.\textsuperscript{119, 120} Due to this limitation, no large-scale glycosylation analysis of IgE in clinical cohorts has been performed as of yet. However, recent advances in LC- and CE-MS sensitivity and robustness may allow for some attempts in the near future.

**Conclusions and Perspectives**

Thanks to the improvement of sample preparation methods and analytical technologies, recent years have seen an increase in sensitivity, accuracy and robustness of IgG glycosylation analysis. These methodological and technological advances are beneficial for biopharmaceutical companies, allowing a better characterization of antibody-based biopharmaceuticals, biosimilars and bio-betters, but are also crucial tools in both basic and clinical research. Thus, this enables, among others, the characterization of glycosylation of antigen-specific IgG, including autoantibodies, alloantibodies and some anti-pathogen antibodies, which directly impact the immune response and the outcome, progression and/or severity of diseases.\textsuperscript{19, 36, 89, 91-95, 99} Therefore, methodologies and technologies dedicated to IgG glycosylation analysis have great prospects regarding the early detection and diagnostic of some diseases. Of note, most studies on IgG glycosylation have focused on serum/plasma antibodies, while IgG in other biofluids and tissue remain largely unstudied.

In addition to IgG, the substantial recent advances in purifying and analyzing small amounts of samples have helped to analyze the glycosylation of other immunoglobulin subclasses (i.e. IgA, IgM and IgE) in a more precise and comprehensive manner. Today’s technological level allows for the simultaneous analysis of multiple immunoglobulin classes in one run.\textsuperscript{60}
We expect that in the near future, several hiatuses in immunoglobulin related glycomics will be covered. Not only by thorough analysis of the glycosylation of all immunoglobulin classes, but additionally by complementary glycoproteomics analysis of many interacting proteins, such as cell surface derived Fc receptors. This may reveal a regulatory role of both antibody and receptor glycomic variation and the interaction thereof in the regulation of antibody effector functions.  

**Acknowledgements**

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CHAPTER 3

IgG Fab glycosylation analysis using a new mass spectrometric high-throughput profiling method reveals pregnancy-associated changes


Abstract

The N-linked glycosylation of the constant fragment (Fc) of Immunoglobulin G has been shown to change during pathological and physiological events and to strongly influence antibody inflammatory properties. In contrast, little is known about Fab-linked N-glycosylation carried by approximately 20% of IgG. Here we present a high-throughput workflow to analyze Fab and Fc glycosylation of polyclonal IgG purified from 5 μL serum. Thirty-seven different N-glycans could be detected and quantified by MALDI-TOF-MS analysis in reflectron positive mode using a novel linkage specific derivatization of sialic acid. This method was applied to 174 samples of a pregnancy cohort to reveal Fab glycosylation features and their change with pregnancy. Data analysis revealed marked differences between Fab and Fc glycosylation, especially in the levels of galactosylation, sialylation, incidence of bisecting GlcNAc and presence of high mannose structures, which are all higher in the Fab portion compared to Fc, while Fc showed higher levels of fucosylation. Additionally, we observed several changes during pregnancy and after delivery. Fab N-glycan sialylation was increased and bisection decreased as compared to post-partum time points, while nearly complete galactosylation of Fab glycans was observed throughout. Fc glycosylation changes were similar to results described before, with increased galactosylation and sialylation and decreased bisection during pregnancy. We expect that the parallel analysis of IgG Fab and Fc, as set up in this paper, will be important for unraveling roles of these glycans in (auto)immunity, which may be mediated via recognition by human lectins or modulation of antigen binding.
Introduction
Immunoglobulins are key players of the human immune system. Immunoglobulin G (IgG) is the most abundant representative of this group, with serum concentrations of approximately 10 mg/mL.\(^1\) It consists of two heavy chains (\(\gamma\)-chains) made up of three constant regions (\(C_\gamma1\), \(C_\gamma2\) and \(C_\gamma3\)) and one variable region (\(V_\gamma\)). Attached to each heavy chain is a light chain (\(\lambda\) or \(\kappa\)). Based on chemical and biological properties, different regions can be distinguished in the IgG molecule: two antigen binding fragments (obtained as F(ab’)\(_2\) by IdeS treatment; further referred to as Fab) and a fragment crystallizable (Fc). The structure of IgG is schematically presented in Figure 1.

IgGs are glycoproteins, and \(N\)-glycans are present at Asn297 of the \(C_\gamma2\) domain. These glycans consist of a constant heptasaccharide core that is often modified by a core fucose, and is in part decorated with bisecting \(N\)-acetylglucosamine (GlcNAc), galactose(s) and sialic acid(s) (Fig. 1).\(^1\) The Fc glycans have been extensively studied, and glycosylation changes have been found to be associated with disease, e.g. rheumatoid arthritis\(^2,3\), and aging.\(^4-6\) Several immune regulatory properties have been demonstrated for IgG Fc glycans.\(^7-13\) For example, Fc-linked glycans influence the IgG effector function by altering the 3D structure of the protein, and thereby the binding to Fc\(\gamma\)-receptors (Fc\(\gamma\)Rs).\(^12,13\) Additionally, glycan-glycan interactions occur between IgG and the Fc\(\gamma\)R-IIIa\(^8\), with the presence of a core fucose decreasing this affinity by approximately 2 orders of magnitude.\(^7\)

The Fab portion consists of the heavy chain \(C_\gamma1\) and \(V_\gamma\) region combined with a light chain, and exhibits the antigen binding sites formed by the variable and hypervariable regions of those two chains. \(N\)-glycans are known to occur on 15-25\% of the IgG Fab portions.\(^1,14,15\) The Fab \(N\)-glycans can be involved in immunomodulation, since they influence the affinity and avidity of antibodies for antigens\(^16-19\), and antibody half-life.\(^17,20\) The glycans of the Fab have been described to be biantennary complex type structures that are, in contrast to Fc glycans, highly sialylated.\(^21-23\) Additionally, high mannose type structures have been described to be located on the Fab portion.\(^23\)

Figure 1. Schematic representation of IgG with the heavy \(\gamma\) chains (dark blue), light chains (lighter blue), and \(N\)-glycans. In the right top corner of the Fc and Fab areas the percentages of galactosylation, sialylation, bisection, and fucosylation are depicted. The inset represents the stable heptasaccharide core with possible extensions.
Pregnancy is known to be associated with overall changes on IgG glycosylation. Indeed, a marked increase of galactosylation and sialylation has been observed on IgG Fc glycosylation during pregnancy. In addition, lectin binding studies suggest changes in Fab glycosylation of IgG during pregnancy, which may be caused by increased levels of progesterone. Changes in glycosylation during pregnancy could be one of the mechanisms that contribute to acceptance of the fetal allograft by the maternal immune system.

Our knowledge on the Fab glycosylation of IgGs from peripheral blood is scarce, which is in part due to difficulties detecting the glycans in a Fab-region specific manner. Due to the polyclonal nature of serum IgG, one may expect Fab glycans to be attached to a large variety of sequence motifs arising from somatic rearrangements and mutations, making the analysis of Fab glycopeptides from polyclonal serum IgG very demanding, if feasible at all. Therefore, studying the Fab glycosylation of polyclonal serum IgG has mainly been pursued at the level of released glycans. Difficulties lie in the purification of IgG, and the separation of Fc and Fab glycosylation, which is essential for the assignment of the glycans to either part of the IgG molecule.

Here we present a high-throughput method for studying Fab glycosylation at the level of released glycans obtained from serum derived polyclonal IgG. Using state-of-the-art affinity capturing beads and enzymes we were able to obtain Fab and Fc separately, which after glycan release resulted in Fc and Fab-specific glycan pools. The released glycans were subjected to a novel derivatization protocol resulting in linkage-specific modification of sialic acids, followed by HILIC sample purification and MALDI-TOF-MS. Finally, since marked changes in glycosylation have been described during pregnancy, the technique was applied to consecutive serum samples from a cohort of pregnant women. This approach was chosen to determine the usefulness of this technique in a clinical setting. The method proved to be able to demonstrate pregnancy related changes in glycosylation of the Fab portion, in addition to the already known changes in Fc glycosylation.

Experimental procedures

Chemicals used
Ethanol, trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), disodium hydrogen phosphate dihydrate (Na₂HPO₄ x 2H₂O), hydrochloric acid (HCl) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Hydroxybenzotriazole (HOBT) hydrate, 50% sodium hydroxide (NaOH) and Nonidet P-40 (NP-40) were obtained from Sigma-Aldrich. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) hydrochloride originated from Fluorochem (Hadfield, UK). Peptide:N-glycosidase F (PNGase F) was bought from Roche Diagnostics (Mannheim, Germany), 2,5-dihydroxybenzoic acid (DHB) from Bruker Daltonics (Bremen, Germany) and HPLC SupraGradient ACN from Biosolve (Valkenswaard, Netherlands). MQ (Milli-Q deionized water; R > 18.2 MΩ cm⁻¹; Millipore Q-Gard 2 system, Millipore, Amsterdam, The Netherlands) was used in this study.
IgG purification
IgG was captured from 5 µL serum, diluted in 100 µL PBS, using 10 µL CaptureSelect IgG-Fc (Hu) beads (Life Technologies Europe, Bleiswijk, The Netherlands) in a 96-well format on an Orochem filter plate (10 µm pore size; Orochem Technologies, Naperville, IL). Alternatively CaptureSelect IgG-CH1 beads (Life Technologies Europe) or Protein G-Sepharose Fast Flow beads (GE Healthcare, Uppsala, Sweden) were used. The beads were washed 3 times with 200 µL PBS on a vacuum manifold before the serum was applied to the beads. Application of the samples to the beads was followed by a 60 min shaking step at room temperature on a multiwell plate shaker with 1.5 mm orbit at 1000 rpm (VWR, Amsterdam, The Netherlands). After removal of the diluted serum, the beads were washed twice with PBS and twice with digestion buffer (50 mM NaH$_2$PO$_4$/150 mM NaCl; pH 6.6).

On-bead FabRICATOR digestion
IgG was specifically cleaved into Fc and Fab portions by recombinant streptococcal IdeS enzyme (tradename FabRICATOR; Genovis, Lund, Sweden). The supplier’s protocol was adjusted to simplify our procedure and reduce costs. We used 10 U enzyme per 50 µg sample (IgG captured from 5 µL of serum, assuming an IgG serum concentration of 10 mg/mL), as compared to 50 U for 50 µg suggested by the supplier. To each sample 35 µL digestion buffer was added, containing 10 U of the enzyme. The Fc portions remained attached to the beads, while the flowthrough, containing Fab fragments, was collected by centrifugation (1 min, 50×g) into V-bottom plates (Greiner Bio-One, Frickenhausen, Germany) after overnight incubation at 37°C in a humidified environment. Following the washing steps with 3× 200 µL PBS and 3× 200 µL MQ, the Fc portions were eluted using 100 µL 100 mM HCl, and collected into V-bottom plates containing 20 µL 500 mM NaOH to neutralize the elution liquid and prevent the loss of sialic acids due to the acidic environment. Subsequently, both Fc and Fab samples were dried by vacuum centrifugation.

LC-MS/MS analysis of IgG, Fc and Fab samples
To further investigate the sample purity, proteomics analysis was performed on the Fab containing flowthrough, and Fc or IgG containing eluates. The samples were dried in the vacuum centrifuge and reconstituted in 40 µL 25 mM ammonium bicarbonate with 1 µL 200 mM DTT. After 1h reduction at 60 °C, alkylation was performed with 3 µL 220 mM iodoacetamide, and sequentially followed by quenching with DTT and overnight digestion with trypsin (1:50 enzyme:protein ratio). LC-ion trap MS/MS analysis was performed as described previously. Peak lists were generated using Data analysis 4.0 (Bruker Daltonics, Bremen, Germany) with default settings and exported as Mascot Generic Files. Peptides were identified in the uniprothuman 20131211 database (88473 sequences; 35069569 residues) using the Mascot algorithm (Mascot 2.4.1, Matrix Science, London, UK) applying a combined search in Mascot Deamon 2.2.2. A peptide mass tolerance of ±0.5 Da (with # 13C=1) and a MS/MS fragment tolerance of ±0.5 Da were used. Trypsin was designated as
the enzyme and up to one missed cleavage was allowed. Carbamidomethylcysteine was selected as a fixed modification and oxidation of methionine as a variable modification. Proteins were considered to be truly detected when hits with at least two unique peptides with a score above 30 were observed.

**N-Glycan release and sialic acid ethyl esterification**
To release the N-glycans from both Fc and Fab 10 µL PBS and 20 µL 2% SDS were added to the dried samples, followed by a 15 min incubation at 60°C. PNGase F (0.25 U per sample) was added in 20 µL of a 5× PBS/4% NP-40 (1:1) solution and the samples were incubated overnight at 37°C in an desiccator.
The following day, 20 µL of the sample was added to 100 µL 250 mM EDC / 250 mM HOBT in pure ethanol. Ethyl esterification of sialic acids was performed at 37 °C for 1h, as described before.\(^{31}\)

**HILIC purification of N-glycans**
The ethyl esterified samples were prepared for cotton HILIC purification by addition of 100 µL acetonitrile, followed by a 15 min incubation at -20°C to precipitate proteins. As a modification of the original cotton HILIC microSPE method \(^{32}\), a piece of cotton thread was used for glycan purification, as detailed in the following: for preparation of the HILIC microSPE devices, twenty μL pipette tips (Rainin Instrument, Oakland, US) were packed with 3 mm cotton thread (180 μg, Pipoos, Utrecht, Netherlands), which was then conditioned and equilibrated by pipetting three times 20 μL MQ, followed by three times 20 μL 85% ACN. The sample was then loaded on the cotton by pipetting up-and-down 20 times. Finally, the cotton was washed 3 times with 20 μL 85% ACN containing 1% TFA and three washes with 20 μL 85% ACN, followed by elution in 30 µL MQ.

**MALDI-TOF MS measurement**
One microliter of the glycan eluate was mixed on spot with 5 mg/mL DHB in 50% ACN containing 1 mM NaOH on a Bruker AnchorChip plate (part nr. 209514, 800 μm anchor; Bruker Daltonics, Bremen, Germany) and allowed to dry at ambient temperature. Shortly before measurement, samples were recrystallized with 0.2μL EtOH. Measurement was performed using an UltraflexXtreme MALDI-TOF mass spectrometer (Bruker Daltonics) in reflectron positive mode. Automated measurement was performed in flexControl 3.4 Build 119. Random walk through the complete sample was used, 20000 shots per walk, 200 shots per step at 2000 Hz. Summed spectra were saved.

**Data processing**
The mass spectrometer was externally calibrated using peptide calibration mix (Bruker Daltonics). The obtained spectra were internally calibrated in flexAnalysis 3.3 Build 80 using a stepwise calibration with initial calibration on the following glycan species ([M+Na]'):
H3N4F1 (m/z 1485.534), H4N4F1 (m/z 1647.587), H4N5F1 (m/z 1850.666), H5N4E1 (m/z 1982.708), the 2nd isotopic peak of H5N4F1E2 (m/z 2448.896) and the 2nd isotopic peak of H5N5F1E2 (m/z 2651.975), with 4 m/z peak picking window using the Snap algorithm. The initial calibration was followed by calibration on 5 different glycan peaks ([M+Na]+) with a window of 1 m/z: H5N4 (m/z 1663.582), H5N4F1 (m/z 1809.640), H5N4F1E1 (m/z 2128.766), H5N4E2 (m/z 2301.835), and H5N5F1E1 (m/z 2331.845) (H, hexose; N, N-acetylhexosamine; F, fucose; E, ethyl esterified N-acetylneuraminic acid). These 11 calibrants cover the most abundant glycans of total IgG, Fc and Fab glycan spectra. Calibrated spectra were exported as text files, containing all data points with an m/z value and intensity. Internal calibration and export was performed using the flexAnalysis Batch Process (Bruker Daltonics). Using an in-house developed script for Python the spectrum was integrated at the m/z ranges that were calculated based on the theoretical glycan list of 42 theoretical N-glycan compositions. Processing in Python took about 4 seconds per spectrum. Output was saved as a text file that could be opened with Microsoft Excel 2010 for further analysis.

Of the 42 potential glycans, thirty-seven N-glycan compositions were mostly observed with signal-to-noise ratios above 3 (supplemental Table S1). The relative abundance of the glycans was calculated after normalization to a total of 100%. For further analysis, the spectra had to pass a quality control set in Excel. Bad spectra were removed by setting cut-off criteria. More than 95% of the spectrum had to be explained by glycan peaks with a signal-to-noise ratio of over 3. Additionally, the extracted data should have >500,000 total signal for all picked glycans after background subtraction. The glycan data were furthermore used to calculate global glycosylation traits: galactosylation, abundance of neutral, mono- or disialylated (S1 and S2, resp.) glycans, fucosylation, bisection, abundance of high mannose structures or overall percentage of α2,3- and α2,6-linked sialic acids. The calculations are shown in the supplemental information. Of note: the sum of α2,3 and α2,6 is not the same as the sum of S1 and S2, since only one of two possible sialylation sites of S1 glycans contains a sialic acid.

Application to clinical samples
Sera from 29 healthy Caucasian women without adverse obstetric history were obtained at three time points during pregnancy and three time points after delivery. The individuals participated as a reference group in the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study. The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands. The last time point (range 26-52 weeks post-partum) after delivery was added in a later stage of the study, and therefore the spread in ‘weeks after delivery’ is larger than for the other time points, namely 26-52 weeks. The above described method was applied to 174 serum samples from the cohort, 9 standard serum samples, and 9 blanks. In total 576 spectra were obtained. The relative abundance of the individual glycans, and the calculated glycosylation traits were evaluated for these samples. Statistical analyses were performed using the ‘signrank’ function in Stata/SE 13.0 for Windows.
The Wilcoxon sign-ranked test was used to test pairwise differences between Fc and Fab glycans, Fc and Fab glycosylation traits, and changes within Fc or Fab glycosylation over time.

Figure 2. Workflow of IgG Fab and Fc glycosylation analysis, including an SDS-PAGE gel confirming IdeS digest. IgG is captured from serum, digested overnight, and the flowthrough and eluate are collected. After drying, the glycans are released from the separate samples, derivatized and purified before measuring on the MALDI-TOF.
Results

High-throughput preparation of polyclonal IgG Fab and Fc portions

To allow the analysis of released N-glycans from IgG Fab and Fc high protein purity has to be obtained. Additionally Fab and Fc have to be separated properly. Here, we developed a high-throughput approach for the analysis of patient cohorts to get more insight in general principles in Fab glycosylation. The final approach presented here was obtained after testing several IgG capturing and cleavage conditions, as well as glycan purification conditions (described in the Supplemental material). The final workflow allowing the generation and the purification of IgG Fc- and Fab fragments from serum is presented in Figure 2. Briefly, IgG was captured from serum or plasma using anti-IgG-Fc beads. The capturing buffer was then replaced by IdeS digestion buffer and the IdeS protease. After overnight digestion the Fab fragment was collected in the flowthrough and the Fc portion in the eluate. A typical SDS-PAGE analysis of the different fractions is depicted in Figure 2. Importantly, no Fc band was observed in the Fab sample. Protein purity was furthermore assessed using LC-MS/MS analysis after in solution tryptic digestion of the fractions, showing only some albumin contamination (supplemental Table S2). Some cross contamination of Fab peptides in the Fc fraction, and vice versa were observed. However, MALDI-TOF-MS spectra of the released glycans from the different fractions (total IgG, Fc and Fab) demonstrated that contamination was only minor, confirming the SDS-PAGE analysis. More specifically, the most abundant Fc glycans (>75% summed relative abundance within the Fc spectra) represented <1% summed relative abundance in the Fab spectra (Figure 3B and C). Overall, the profile spectra were in good agreement with literature data on IgG glycosylation.3,14,23

Derivatization and HILIC purification of released Fab and Fc N-glycans

For high-throughput data gathering, a simple mass spectrometric approach, like MALDI-TOF-MS, is most convenient. To suppress in-source and metastable decay of sialylated N-glycans in MALDI-TOF-MS measurements, a derivatization step was performed. The N-glycans were derivatized by ethyl esterification of α2,6-linked sialic acids and lactonization of α2,3-linked sialic acid residues using a recently developed protocol, which additionally allows to differentiate sialic acid linkages.31

After the derivatization, N-glycan samples were purified by HILIC SPE. To this end, the recently introduced cotton wool HILIC micro-SPE approach 32 was adjusted to make it less laborious, while keeping it compatible with high-sensitivity MALDI-TOF-MS analysis. While in the original method small pieces of cotton wool (approx. 500 µg of cotton wool) are brought into a pipette tip to form a filter-less HILIC stationary phase, we here packed small pieces of cotton thread (approx. 180 µg; 3 mm long) into a pipette tip which made the preparation of the cotton tips easier and faster, while maintaining the high yield of the original cotton wool microtips.32 The total time for preparing the HILIC tips and executing the SPE purification was approximately 45 min for 96 samples (1 plate).
High-throughput analysis of released Fab and Fc N-glycans

To demonstrate the applicability of the developed method, aliquots of ethyl esterified glycans from IgG, Fab and Fc were subjected to cotton thread HILIC micro-SPE, spotted with DHB matrix, followed by recrystallization with ethanol for optimal MALDI shot-to-shot reproducibility. This MALDI-TOF-MS method was applied on 174 serum samples obtained from 29 healthy women during and after pregnancy, as well as on 9 replicates of a standard sample. Glycans observed for total IgG, Fab and Fc of human IgG were assigned on the basis of literature knowledge, and this glycan list was refined based on species observed in a subset of samples. The final list of detected glycans and their calculated masses are depicted in supplemental Table S1.

Glycan compositions as well as major structural features of the most abundant glycans from Fc and Fab spectra were confirmed using MALDI-TOF/TOF-MS/MS (supplemental Fig. S1). IgG, Fab and Fc spectra were internally calibrated, followed by extraction of glycan signals, which were subsequently normalized to a total sum of 100%. Low quality profiles were removed, as judged by total signal intensity and signal-to-noise ratios. Two spectra were excluded for IgG, 2 for Fc, and 24 out of 174 for Fab.

Robustness of the method

Repeatability of the method was tested using a control serum sample along with the test sera. Inter- and intra-plate variation was calculated for the glycans with an average relative abundance of 1% or more over all control samples. In the IgG samples there were 9 major glycans, representing >95% of the glycan signal in the spectrum, with average intra-plate variation of 9%, and 12% inter-plate. For Fc spectra 7 glycans, representing >95% of the total glycan signal, showed average intra-plate variation of 12%, and 18% inter-plate. The Fab spectra contained 11 most abundant signals, representing >93% of the total glycan signal, with an average intra-plate variation of 12%, and 17% inter-plate. Average relative intensities of all extracted glycans and their SD are presented in supplemental Fig. S2, as are the calculated glycosylation traits. In addition, the relative intensities (RI) of the major glycans of IgG, Fc and Fab, and the corresponding relative standard deviations (RSDs) are depicted in supplemental Table S3.
Figure 3. Typical IgG, Fc and Fab mass spectra. Typical MALDI-TOF-MS spectra obtained for the released glycans of total IgG (A), Fc (B), and Fab (C). The zoom-in shows isotopic resolution even for the low abundant peaks.
FC and Fab are differentially glycosylated

Data of the pregnancy time courses were analyzed to reveal glycosylation differences between Fab and Fc as well as pregnancy-associated changes in Fab glycosylation. For the former purpose, we compared the Fab and Fc glycosylation profiles obtained for the last time point (6 months after delivery), since these samples will hardly be affected anymore by the preceding pregnancy, as we observed in the dataset of a previous study on Fc glycosylation and rheumatoid arthritis. Glycosylation patterns were compared after signal extraction and normalization. Levels of galactosylation were on average found to be much higher for Fab (94%) than for Fc (67% Fc, p<0.0001; Table 1). Likewise, the levels of sialylation were much higher for Fab as compared to Fc: monosialylated species were 40% for Fab versus 18% for Fc (p<0.0001). For disialylated species the differences were even more pronounced, with 52% for Fab and less than 1% for Fc (p<0.0001). Additionally, there is a difference in the sialic acid linkages. On the Fab 1.9% α2,3-linked sialylation was observed, while only trace amounts (0.1%) of α2,3-linked sialic acids were found in the Fc fraction (p<0.0001). In line with the presence of sialylated glycan species, the percentage of neutral glycans was much lower for Fab (7%) than for Fc (81%; p<0.0001). Levels of bisection were higher for Fab (45%) than for Fc (10%; p<0.0001). Interestingly, while Fab glycans were high in galactosylation, sialylation and bisection, they were low in fucosylation as compared to (69% vs. 94% for Fc; p<0.0001).

Pregnancy associated changes in Fab glycosylation

In addition to the comparison between Fab and Fc glycosylation, changes in time within the different IgG portions were analyzed. The changes in time of the mean levels of glycosylation traits with pregnancy and after delivery are given in Fig. 4 and Table 2, and data distribution is visualized using boxplot representation in supplemental Fig. S3. Additionally, for all the individual observed glycans, the relative abundances at the different time points are

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<td>9.60 0.38</td>
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presented in supplemental Fig. S4, and the relative abundances at 6 months after delivery are numerically depicted in supplemental Table S1, along with the standard deviations. The observations presented in Fig. 4 appear similar for total IgG and Fc, although total IgG levels are increased for glycosylation traits that are high on the Fab portion, for example Fig. 4D.

First, we looked at changes of IgG Fab and Fc glycosylation with delivery. On the Fab portion increased levels of monosialylated glycans were detected 6 weeks after delivery (41%) compared to the third trimester of pregnancy (37%; p<0.0001; Fig. 4C), at the expense of Fab disialylation (56% to 51%; p<0.0002; Fig. 4D). The total percentage of both α2,3- and α2,6-linked sialic acids decreased after delivery for Fab and Fc (Fig. 4H, I). In addition, increases after delivery were observed for fucosylation (67% in the third trimester to 70%; p<0.002; Fig. 4F). Notably, bisection showed a rather prominent increase with delivery (from 37% to 45%; p<0.0002; Fig. 4E). A decrease was detected within 6 weeks after delivery for Fc galactosylation (77% to 69%; p<0.0001; Fig. 4A) and monosialylation (25% to 19%; p<0.0001). The sialic acids were mainly α2,6-linked. Increased levels of α2,3-linked sialic acids were observed on Fab in comparison with Fc. Neutral Fc glycans were found to be relatively more abundant 6 weeks after delivery (80%) compared to the third trimester of pregnancy (75%; p<0.0001; Fig. 4B). Fc fucosylation was already high during pregnancy and showed a slight increase with delivery (94% to 95%; p<0.0002). Additionally, the presence of a bisecting GlcNAc on Fc glycans is increased after delivery (10%) in comparison to the last time point during pregnancy (8%; p<0.0001). Hence, these comparisons established clear and consistent differences in Fab and Fc glycosylation between pregnancy (3\(^{rd}\) trimester) and the non-pregnant status (6 weeks after delivery). No major difference were observed between the three post-pregnancy time points (6 weeks, 3 months and more than 6 months after delivery (Figure 4).

Next, we looked at glycosylation changes occurring in the time course of pregnancy. For this, we analyzed changes in IgG Fab and Fc glycosylation between the 1\(^{st}\) and 3\(^{rd}\) trimester of the pregnancy. Regarding the Fab portion, no obvious glycosylation changes were observed, except perhaps for a minor albeit significant decrease in monosialylated (from 38% in the first trimester to 37% in the third trimester; p<0.003) and bisected glycoforms (39 to 37%; p<0.03), and the percentage of α2,3-linked sialic acid showed a minor increase during pregnancy. In contrast, for the Fc, more pronounced changes in glycosylation was observed. An increase in the level of galactosylation was observed between the first (74%) and third trimester (77%; p<0.0001). Similarly an increase in the level of monosialylation was observed when comparing first (23%) and third trimester (25%; p<0.002). Glycans on the Fc portion appear to be less fucosylated in the 3\(^{rd}\) trimester (94.1%) relative to the 1\(^{st}\) trimester (94.5%; p<0.03). Likewise, neutral glycosylation is decreased in the third trimester (75%) in comparison with the first (76%; p<0.002). The abundance of high mannose structures did not change significantly for neither Fab nor Fc (Fig. 4G).
Figure 4. Timelines representing glycosylation variation during and after pregnancy. Various differences in glycosylation between pregnancy and after delivery time points can be observed for total IgG (left), Fc (middle) and Fab (right) with respect to galactosylation (A), neutral (B), mono- and disialylated glycans (C, D), incidence of bisecting GlcNAc (E), fucosylation (F), the abundance of high mannose structures (G), and α2,3- (H) and α2,6-linked (I) sialic acids. Error bars represent the standard error of the mean. Abbreviations: trim, trimester of pregnancy; wk, weeks; pp, post-partum.
Table 2. Glycosylation changes during pregnancy (1st vs. 3rd trimester) and immediately after delivery (3rd trim vs. 6 wk pp).

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* Wilcoxon sign-ranked test p-values <0.05 are highlighted in bold and considered to be significant. The statistical test was used to make a pairwise comparison of the 1st and 3rd trimester (trim1 and trim3, respectively), and of the 3rd trimester vs. 6 weeks post-partum (6wkpp).
Discussion
Here we present a high-throughput profiling method for analyzing the glycosylation of both the constant and the variable region of polyclonal serum immunoglobulin G in a straightforward workflow. We applied the method to compare Fab and Fc glycosylation profiles of young women, and to study pregnancy-associated changes in IgG Fab and Fc glycosylation in the same group. The analysis of Fc released glycans using the method described in this manuscript yielded results that were highly comparable to those previously obtained for the same sample set by LC-MS. In addition, this approach for the first time provided high-quality Fab glycosylation data of a clinical cohort.

Glycosylation analysis of the Fab domain of IgG has been performed before, but mainly on monoclonal antibodies. To our knowledge only few papers describe the analysis of the Fab glycosylation of polyclonal IgG derived from peripheral blood, and only very limited numbers of sera were studied leaving the nature of human polyclonal IgG Fab glycans and their biological variation largely obscure. Additionally, Agglutinin and concanavalin A (ConA) assays provided inconclusive insights into the molecular nature of human Fab glycosylation. In contrast to these lectin based assays, the method described in this paper uses mass spectrometric detection of IgG derived N-glycans, which in combination with an optimized method for the preparation of Fc and Fab portions provides detailed information on relative abundances, compositions and major structural features of IgG Fab and Fc N-glycans. Obviously, the mass spectrometric approach for detection of released glycans is relative by nature, and no absolute quantitation results can be obtained. Furthermore, due to the normalization on total intensity, relative abundances are reported throughout.

We used an innovative on-bead digestion preventing the need for additional purification procedures to obtain separate Fc and Fab samples. The IdeS enzyme has been used before for the generation of Fc and Fab portions and their glycosylation analysis, but mainly on monoclonal antibodies. IdeS is known to cleave only IgG at a specific cleavage site, leaving both Fab and Fc intact. Alternative enzymes come with some disadvantages, like a varying digestion site or multiple digestion sites in the Fc portion. The ethyl esterification of sialic acids used in this study allowed for sensitive detection of glycans using the MALDI-TOF-MS in reflectron positive mode. This resulted in high resolution spectra, without the loss of sialic acids that is common in RP MALDI measurements. We registered levels of Fc-sialylation similar to those determined by LC-MS, while without stabilization, sialic acids exhibit low relative abundances in MALDI-TOF-MS experiments. In addition, the sialic acid ethyl esterification results in linkage specific mass differences. This allowed the facile differentiation of α2,3- and α2,6-linked sialic acids in a high-throughput fashion.

Analysis of our data confirmed previous reports of the presence of high mannose structures on the Fab portion of IgG. The data indicate higher levels of these glycans than observed by Anumula. Additionally, we observe drastically lower levels of H3N4F1 and H4N4F1.
structures on Fab than in the previous report. Since these glycans may be largely regarded as Fc markers, this may indicate a higher purity of the Fab preparation in our study as compared to the previous one.\(^{23}\) One possible explanation may be that the sequential release of glycans by different enzymes features a less stringent specificity for Fab and Fc than indicated.\(^{23}\)

We analyzed 174 serum samples of 29 individuals covering 6 time points during and after pregnancy. This way we have been able to observe several changes over time (Fig. 4). Biological variation seems to be bigger on the Fab portion compared to Fc, as is reflected by increased standard errors which could not be contributed to technical variation. By applying this method, we demonstrated various changes in Fab glycosylation with pregnancy.

The calculated glycosylation traits show a different behavior for Fc and Fab. For example, although different levels are observed for Fc galactosylation during pregnancy compared to 6 weeks after pregnancy, this does not occur for the Fab galactosylation. Similarly, although to a lesser extent, Fab fucosylation does change after delivery, while Fc fucosylation remains at very similar levels throughout. Furthermore, for monosialylation opposite changes are observed. Monosialylated structures are increased on the Fc portion during pregnancy, and decrease after delivery. On the other hand, Fab monosialylation is decreased during pregnancy compared to the time points after delivery, which may reflect increased turnover of Fab mono- into disialylated species. However, for both Fab and Fc glycosylation the level of bisection decreases during pregnancy, and total sialylation increases.

In contrast to the Fc glycosylation, limited information is available with regards to Fab glycosylation function. In fact, most of the data has been obtained over the last years. Initially, it was shown that the anti-inflammatory properties of IVIG (intravenous immunoglobulins) were due to sialylation of the Fc portion.\(^{46}\) However, an increasing body of evidence suggests that the Fab portion is involved.\(^{47,48}\) Lectins like Siglecs (Sialic acid-binding immunoglobulin-type lectins) and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), recognizing one of the sugar moieties on either one of the IgG portions, may or may not\(^{11,47}\) be involved in these processes. In addition to the influence of Fab glycans to cellular receptors, the glycans may also be involved in modulating antigen binding and antibody half-life.\(^{17,20}\)

In literature increased ConA reactivity during pregnancy has been described.\(^{26,38,39}\) This is generally interpreted as an increase in Fab glycosylated -also called asymmetrical-antibodies, which occurs under the influence of progesterone\(^ {38}\), among other factors. In \textit{in vitro} settings the increase in ConA reactivity has been confirmed by some\(^ {27}\), while others observed similar increases in ConA reactivity with low progesterone concentrations, whereas high concentrations resulted in the contrary.\(^ {51}\) The increase of ConA reactivity is believed to reflect increased levels of high mannose glycans only present on the Fab portion, excluding interference from the Fc portion (Asn297), which is known to bear hardly any oligomannosidic glycans.\(^ {2,6,35,52}\) However, ConA has also been reported to have affinity for non-bisected glycan structures\(^ {41}\), thereby exhibiting increased binding with decreased levels of bisecting GlcNAc with pregnancy on both Fc and Fab. One may speculate that the
glycosylation changes observed in our study are likewise caused by hormonal changes, yet more studies are needed to reveal how the cellular glycosylation machinery is regulated by hormones.

In conclusion, we developed a high-throughput method enabling the separate detection of glycans derived from human polyclonal IgG Fab and Fc. When this technique was applied to consecutive serum samples from a cohort of pregnant women, it revealed that there are clear differences between Fc and Fab glycosylation of immunoglobulin G. In addition this technique proved to be suitable in demonstrating pregnancy associated changes in glycosylation not only for Fc but also for Fab.

**Acknowledgements**

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**Supplemental material**

The Supplemental material to this manuscript is available at http://www.mcponline.org/content/suppl/2014/07/08/M114.039537.DC1/mcp.M114.039537-1.pdf
References


CHAPTER 4

LONGITUDINAL MONITORING OF IMMUNOGLOBULIN A GLYCOSYLATION DURING PREGNANCY BY SIMULTANEOUS MALDI-FTICR-MS ANALYSIS OF N- AND O-GLYCOPEPTIDES

Albert Bondt, Simone Nicolardi, Bas C. Jansen, Kathrin Stavenhagen, Dennis Blank, Guinevere S.M. Kammeijer, Radoslaw P. Kozak, Daryl L. Fernandes, Paul J. Hensbergen, Johanna M.W. Hazes, Yuri E.M. van der Burgt, Radboud J.E.M. Dolhain, Manfred Wuhrer

Abstract

Immunoglobulin A (IgA) is a glycoprotein of which altered glycosylation has been associated with several pathologies. Conventional methods for IgA N- and O-glycosylation analysis are tedious, thus limiting such analyses to small sample sizes. Here we present a high-throughput strategy for the simultaneous analysis of serum-derived IgA1 N- and O-glycopeptides using matrix-assisted laser/desorption ionisation Fourier transform ion cyclotron resonance (MALDI-FTICR) mass spectrometry (MS). Six non-fucosylated diantennary complex type glycoforms were detected on the Asn144-containing glycopeptide. Thirteen distinct glycoforms were identified for the Asn340-containing tailpiece glycopeptide, mainly of the diantennary complex type, and low amounts of triantennary glycoforms. Simultaneously with these N-glycopeptides, 53 compositional glycoforms of the hinge region O-glycopeptide were profiled in a single high resolution MALDI-FTICR spectrum. Since many pregnancy associated changes have been recognized for immunoglobulin G, we sought to demonstrate the clinical applicability of this method in a cohort of 29 pregnant women, from whom samples were collected at three time points during pregnancy and three time points after delivery. Pregnancy associated changes of N-glycan bisection were different for IgA1 as compared to IgG-Fc described earlier. We foresee further applications of the developed method for larger patient cohorts to study IgA N- and O-glycosylation changes in pathologies.
**Introduction**

Human antibodies consist of two light chains (which can be immunoglobulin kappa or lambda) and two heavy chains (either immunoglobulin alpha, delta, epsilon, gamma or mu). The heavy chains determine the antibody isotypes (IgA, IgD, IgE, IgG or IgM, respectively). For immunoglobulin A two subclasses exist, namely IgA1 and IgA2, as defined by structure differences between the α1 or α2 heavy chains. IgA concentrations in the human circulation are about 2 mg/mL\(^1\), of which IgA1 accounts for approximately 90%. Both IgA1 and IgA2 are post-translationally modified by covalently connected N- and O-glycans (Fig. 1).

Amongst various single amino acid differences, IgA subclasses differ in the hinge region, where the primary sequence of IgA1 contains thirteen additional amino acids (aa) compared to IgA2 (see Supplementary Material S1). Previously, it was found that three O-glycosylation sites in this hinge region were constitutively occupied (Thr106, Thr109 and Ser113), whereas Ser111, Thr114 and Thr117 may or may not carry an O-glycan.\(^2,3\) (In the current manuscript amino acid numbering is based on the constant part or the heavy chains only (UniProt entries: IGHA1/P01876, 353aa; IGHA2/P01877, 340aa), while the referred manuscripts use a numbering based on the traditionally expected total amino acid sequence. Due to the differences in numbering, these sites correspond to Thr225, Thr228, Ser232, Ser230, Thr233 and Thr236 in the referred papers, respectively.) These amino acids carry O-glycans of the core 1 type.\(^2,7\) Such O-glycans consist of a single N-acetylgalactosamine (GalNAc) that can be extended with one galactose (resulting in the T antigen), and up to two sialic acids of which one can be attached to the GalNAc and one to the galactose.

![Figure 1](image.png)

**Figure 1.** Schematic representation of IgA1 showing two N-glycosylation sites on the heavy chain, and hinge-region O-glycosylation. The inset shows several possible core 1 (T-antigen) and Tn antigen O-glycan structures. The ± symbol indicates that the outer monosaccharide may or may not be present.
IgA N-glycans are present at Asn144 and Asn131 in the C\textsubscript{H}2 domain of IgA1 and 2, respectively, and at Asn340 and Asn327 in the tail piece domain.\textsuperscript{8} In addition, IgA2 contains two N-glycosylation sites at Asn47 in C\textsubscript{i,1} and at Asn205 in the C\textsubscript{H}2 domain.\textsuperscript{8} The serum IgA N-glycans have been found to be of the diantennary complex type, with high levels of galactosylation and sialylation, and possible bisection and fucosylation.\textsuperscript{4,9-11} More specifically, the C\textsubscript{H}2 derived glycan of human serum IgA1 was found to be diantennary without a fucose, in contrast to the fucosylated glycan on Asn340.\textsuperscript{11} Others studied polymeric IgA from human colostrum or serum and confirmed the low levels of fucose on the C\textsubscript{H}2 glycan, although the glycans in general were smaller (mainly high mannose, non- or mono-galactosylated complex type).\textsuperscript{12}

It has been found that IgA plays an important role in several pathologies, e.g. IgA nephropathy (IgAN) and Henoch-Schönlein purpura (HSP). One of the main topics of research on IgAN is related to its O-glycosylation, which has been suggested to be involved in IgAN pathogenesis.\textsuperscript{13,14} Moreover, it has been proposed as a marker for therapeutic response in IgAN treatment, with lowered levels of agalactosylated O-glycans as a marker of improvement.\textsuperscript{15} Similarly, the galactose deficient IgA O-glycans have been associated with HSP.\textsuperscript{16} This agalactosylated O-glycan is known as Tn-antigen, and the sialylated form is known as sialyl-Tn-antigen. Rheumatoid arthritis also is associated with changed O-glycosylation of the IgA hinge region, namely lower levels of GalNAc compared to healthy individuals, while the number of galactoses remained the same.\textsuperscript{17}

The role and underlying mechanisms of IgA1 N-glycosylation changes in disease are not yet unravelled. It has been suggested that glycosylation changes influence the binding of IgA to the Fc alpha receptor\textsuperscript{4}, however this was not observed in a later study.\textsuperscript{18} Notwithstanding, binding to Fc\textalpha{}R could induce both pro-and anti-inflammatory processes\textsuperscript{19}, and the role of the N-glycans in driving the process in either one of these directions is unknown. Furthermore, the N-glycosylation may affect binding to receptors responsible for IgA clearance, e.g. asialoglycoprotein receptor.\textsuperscript{20,21} Finally, IgA has been found to bind to the glycan-binding DC-SIGN/SIGN-R1\textsuperscript{22}, which for IgG (in mice) has been suggested to be involved in anti-inflammatory processes.\textsuperscript{23} However, the physiological role of this is still under debate.\textsuperscript{24} Methods reported so far for the detection of IgA glycosylation involve lectin binding arrays, mass spectrometry (MS) in combination with various chromatographic strategies\textsuperscript{2,3,6,18,27-30} and liquid chromatography (LC) with fluorescence detection.\textsuperscript{31} These methodologies lack specificity (lectins) and are time-consuming (LC-MS). Sample throughput can be improved after reducing sample complexity using sialidases,\textsuperscript{3} however at the expense of important information.

MS strategies based on matrix-assisted laser/desorption ionisation (MALDI) allow a high-throughput analysis of a complex mixture with the additional benefit of preventing carryover between samples. Most commonly, MALDI is coupled to time-of-flight (TOF) mass spectrometers. However, for simultaneous MALDI-MS analysis of N- and O-glycopeptides from polyclonal serum IgA high resolving powers (>50,000) are pivotal. Moreover, sialic
acids are instable in commercially available MALDI-TOF systems. Therefore, MALDI has hitherto hardly been applied for IgA O-glycopeptide analysis.\textsuperscript{32} Fourier transform (FT) MS methods provide the highest resolving power and mass accuracy within the different mass spectrometers.\textsuperscript{33} Previously, we have applied MALDI-FT- ion cyclotron resonance (ICR)-MS to map singly-charged proteins up to about 17000 Da and use the accurate mass differences for identification purposes.\textsuperscript{34,35} These properties allow both the resolution of near isobaric species and the identification of possible post-translational modifications (PTMs). Another advantage of MALDI-FTICR-MS is the fact that the intermediate pressure in the MALDI source leads to a significant reduction of the sialic acid fragmentation.\textsuperscript{36} Even though a MALDI approach will not allow for full discrimination of all macro- and microheterogeneity, it is suitable for high-throughput profiling in a clinical setting. Therefore, in this study serum IgA glycopeptides were enriched with high specificity using a combination of purification techniques including a 96-well plate IgA affinity purification, trypsin digestion and two sequential HILIC solid-phase extractions. The enriched N- and O-glycopeptides were measured with isotopic resolution in an m/z-range from 3499 to 10000 using a 15T MALDI-FTICR mass spectrometer. This approach allowed for the first time the specific, high-throughput analysis of both N- and O-glycosylation of IgA1 by ultrahigh resolution MALDI-FTICR-MS. We observed 53 different compositions for the O-glycopeptide, 6 glycoforms for the Asn144 containing N-glycopeptide, and 13 glycoforms for the Asn340 containing glycopeptides. Confident assignment of the glycoforms was achieved making use of the high resolution and mass accuracy, together with fragment ion spectra. For immunoglobulin G it is known that many glycosylation changes occur with pregnancy, and therefore we applied our method on a pregnancy cohort (n=29 individuals, 6 time points) to explore whether the same holds true for immunoglobulin A. The application of this method showed several site specific differences in N-glycosylation between pregnancy and the postpartum state. O-glycosylation showed some minor pregnancy associated alterations with respect to galactosylation and the ratio of galactoses per GalNAc.

Results
IgA capturing and digestion
We established a workflow for the high-throughput profiling of serum IgA1 N- and O-glycosylation by MALDI-FTICR-MS. IgA was affinity-captured from human serum in a 96-well format resulting in a highly enriched IgA sample (see Supplementary Table S1). The captured IgA was digested using trypsin after reduction and alkylation in order to generate specific glycopeptide fragments.

MALDI-FTICR-MS profiling of IgA glycopeptides
To be able to accurately detect all glycoforms present in the IgA glycopeptide mixture ultrahigh resolution MALDI-FTICR-MS measurements were performed using 4-chloro-\alpha-cyanocinnamic acid matrix after HILIC enrichment of the IgA glycopeptides. HILIC
enrichment was optimized to obtain the IgA1 hinge-region \(O\)-glycopeptides and the IgA1 Asn340 \(N\)-glycopeptides in one single spectrum after purification from 70% ACN, while the second IgA1 \(N\)-glycopeptide cluster (Asn144) was subsequently HILIC enriched from the flow-through starting with 80% ACN (see Methods section for the details) and obtained in a second spectrum (Fig. 2).

**Figure 2.** Typical spectra of IgA glycopeptides as obtained by MALDI-FTICR-MS after trypsin digestion and cotton HILIC SPE. Annotated peaks show the \(O\)-glycosylated hinge-region glycopeptide cluster (A) and the Asn340 glycopeptide cluster (non-truncated peptides are indicated by \(+Y\); B) as obtained from a single mass spectrum from the 70% ACN HILIC step, and the Asn144 glycopeptide cluster (C) as obtained from a mass spectrum after 80% ACN HILIC. The assignment was supported by high mass accuracy, as well as LC-MS/MS of glycopeptides and MALDI-TOF-MS of released glycans. Glycopeptides that were not included in the final analysis after analyte curation are depicted in grey. \(H\) = hexose; \(N\) = \(N\)-acetylated hexosamine; \(F\) = fucose; \(S\) = \(N\)-acytelyneuraminic acid; \(\ast\) = sodium adduct; \# = unidentified non-IgA related glycopeptide contaminant; \(\&\) = unidentified non-glycopeptide contaminant.
**Peak list generation**

Manual inspection of the spectra showed no IgA2 specific glycopeptide clusters. For IgA1 several glycoforms were observed for the N-glycopeptides containing Asn144 (peptide moiety \textit{127}\textbf{LSLHRPAEDLLGSEANLTCTLTGLR}_{153}), or containing Asn340 (peptide moiety \textit{332}\textbf{LAGKPTHVNSVMAEVDGTCY}_{353}), and for the hinge region O-glycopeptide (\textit{89}\textbf{HYTNPSQDVTPCPVPSTPSTPSTPSTPSTPSCHPR}_{126}). In addition, some apparent mass deviations were observed, that could be explained by the loss of C-terminal tyrosine from the Asn340 containing glycopeptide, as was previously observed by others\textsuperscript{18,30}, and by oxidation of the methionine in the Asn340 glycopeptides. Furthermore, we did observe some sodium adducts in the spectra, however these were generally minor and therefore not included in the analysis. In some spectra we observed contaminants and these were excluded from further analysis. The high resolving powers (>50,000) that are characteristic of MALDI-FTICR-MS were crucial for simultaneous profiling of the N- and O-glycopeptides in a single spectrum. Moreover, the high mass accuracies (<2 ppm) provided by the system allowed confident assignment of the IgA glycopeptides (Supplementary Fig. S3).

An analyte list containing the observed putative glycopeptides was curated on the basis of overall abundance and QC-values. The identity of some O-glycopeptide species was confirmed by ESI-FTICR-MS/(MS), and N-glycopeptide confirmation was obtained by C18-nanoLC-quadrupole-TOF-MS/MS. The major glycovariants of each glycopeptide cluster were thus identified (Fig. 3, Supplementary Table S2, and Supplementary Fig. S2). Of note, for the O-glycopeptides no site assignment has been performed. The N-glycopeptide moieties were confirmed by performing a glycan release on trypsin digested IgA, and analysing these digests on LC-ESI-MS/MS, followed by a proteomics data analysis where deamidation was set as a variable modification (Supplementary Methods, Supplementary Table S1). Finally, N-glycan structures were confirmed by analysing ethyl esterified released glycans using MALDI-TOF-MS (Supplementary Methods, Supplementary Fig. S4), and O-glycan structures by HPLC.\textsuperscript{7} The released N-glycan analysis additionally indicated that the sialic acids were almost exclusively \(\alpha2,6\)-linked.

The combined MS and MS/MS data for both glycopeptides and glycans furthermore indicated the absence of difucosylated species, which would, if present, in part overlap with sialylated species. Most of the analysed samples showed a partial oxidation of the glycopeptides covering site Asn340. While the assignment of most of the peaks was straight-forward, oxidized, monofucosylated species, which are isomeric with species that instead contain an additional hexose, were slightly more challenging. However, by using the released glycan information in most cases unambiguous assignment was achieved, which was illustrated by highly similar oxidation ratios for all observed oxidized/non-oxidized glycopeptide pairs (data not shown).
Altogether, this resulted in a final list of 53 O-glycopeptide conformations, 6 glycoforms for the Asn144 containing N-glycopeptide, and 13 glycoforms for the Asn340 containing glycopeptides, of which 3 glycoforms were observed for both the truncated and the non-truncated peptide variant, and 10 only for the truncated peptide (Table 1; Supplementary Table S3). For the O-glycopeptide cluster (approximately m/z 5350-8000) an average mass measurement error after internal calibration of 1.72 ppm was obtained. The N-glycopeptide clusters (approx. m/z 3900-5350) similarly showed low- to sub-ppm average mass measurement errors: Asn340, 0.94 ppm; truncated Asn340, 1.28 ppm; Asn144, 1.37 ppm.

Figure 3. Exemplary ESI-QTOF-MS/MS fragmentation spectrum of an Asn340 non-truncated tryptic glycopeptide [M+4H]4+ of m/z 1179.2420. The spectrum was acquired in combined lower- and higher-energy CID, exhibiting glycan fragmentation (upper panel) and also fragmentation of the peptide backbone (zoom-in lower panel).
Table 1. Detected O-glycopeptides with the corresponding monoisotopic theoretical mass and median observed ppm error. Additionally literature references are depicted for previously observed glycoforms. Abbreviations: H = hexose; N = N-acetylhexosamine; S = N-acetyleneuraminic acid; n.d. = not detected.

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**MALDI-FTICR-MS spectrum selection**

The glycopeptide clusters were subsequently subjected to a quality check (see Methods section for details). Robustness of the method was then tested using a standard plasma sample along with the clinical samples. The standard samples were included at least in triplicate on each 96-wells plate. For all glycopeptide clusters the inter plate variation of the standard was below 20% (Supplementary Table S4), while the biological variation was on average almost twice as high.

After spectra curation the final dataset contained 329 hinge region O-glycosylation profiles, 292 Asn144 N-glycosylation profiles, and 329 Asn340 N-glycosylation profiles. When two profiles of the same glycopeptide cluster were available for a sample, the highest intensity variant was used for the analysis. Only patients for whom spectra of all time points were available were included in the final statistical analysis. Calculated O-glycosylation traits could be analysed pairwise for 27 individuals, traits from Asn144 for 25 individuals, and from Asn340 glycopeptides the traits could be analysed pairwise for 23 (non-truncated peptide) and 25 individuals (truncated peptide).

**Site specific changes in IgA N-glycosylation during pregnancy and after delivery**

The level of bisection of the diantennary glycans on Asn340 increased during pregnancy (truncated peptide: 52% to 54%, \( p = 0.0001 \), Fig. 4A; non-truncated: 52% to 55%, \( p = 0.0072 \), Fig. 4B). Notably, the increase of bisection observed during pregnancy continued after delivery, and highest bisection levels were observed at 6 weeks postpartum (58% as compared to 55% in the 3rd trimester; \( p = 0.0002 \)). Similarly, Asn144 bisection showed its maximum at 6 weeks postpartum (28%, Fig. 4C).

Sialylation of Asn144 was higher during pregnancy than after delivery (63% vs. 59%, \( p = 0.0002 \); Table 2, Fig. 4E). The decrease occurred rapidly between the last time point of pregnancy and 6 weeks postpartum, after which it remained lowered. In contrast, for Asn340 no changes in the level of sialylation (>89%) were observed.

The tailpiece glycan fucosylation showed a minor, but significant, decrease during pregnancy, and an increase after delivery (\( p < 0.002 \), Fig. 4G). This was not reflected on the Asn144 N-glycosylation site, where no fucosylated glycans were observed.

The larger variety of N-glycans on Asn340 compared to Asn144 was further illustrated by the presence of triantennary glycans. The abundance of these glycans increased during pregnancy (5 to 6%, \( p = 0.0017 \), Fig. 4H). After delivery the levels dropped to 5% (\( p < 0.008 \)). Both N-glycosylation sites of IgA1 showed complete or near complete levels of galactosylation, for which no changes were observed during pregnancy, nor after delivery.

Values for all calculated N- and O-glycosylation traits at all time points, and the corresponding standard errors, are depicted in Supplementary Table S5.
Pregnancy associated changes in IgA O-glycosylation

The O-glycosylation of the IgA1 hinge region remained fairly stable throughout pregnancy and the time after delivery. The numbers of GalNAc residues and sialic acids did not change, and neither did the number of sialic acids per galactose. Very small yet significant increases of the number of galactoses (3.96 to 3.98, \( p = 0.0095 \); Table 2, Fig. 4I) and the related ratio of galactoses per GalNAc (0.82 to 0.83, \( p = 0.0109 \), Fig. 4J) during pregnancy were observed.

Figure 4. Observed IgA glycosylation changes over time during pregnancy and after delivery for the calculated traits. IgG data is included for comparative reasons (taken from Ref. 38). N-glycosylation: levels of bisecting GlcNAc (A-C; comparison with IgG in D), sialylation (E; comparison with IgG in F), and fucosylation (G) on diantennary complex type glycans; level of triantennary glycans (H). O-glycosylation: number of galactoses per peptide (I) and the ratio of Galactoses per GalNAc (J). Depicted are the mean values per time point, error bars represent standard errors.
Table 2. Mean and standard error of the calculated glycosylation traits for the time points used in the statistical analysis. In addition the number of paired samples for the Wilcoxon signed-rank test are depicted, as well as the resulting p-values. P<0.0167 was considered as statistically significant after Bonferroni correction.

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*Abbreviations: trim = trimester; wkpp = weeks postpartum; GalNAc = N-acetylgalactosamine; Gal = galactose; SA = N-acetylneuraminic acid.
Discussion

In the current manuscript we describe a method for the simultaneous analysis of IgA1 N- and O-glycosylation using affinity purification and a simple trypsin digestion, followed by a two-step HILIC enrichment of the glycopeptides, and MALDI-FTICR-MS detection. The high-throughput technique is estimated to allow for the analysis of 384 serum samples within 24 h. Fifty-three O-glycopeptide compositions, 6 Asn144 glycoforms and 13 Asn340 glycoforms can be distinguished with high mass accuracy (average <2 ppm error). The technique was applied on a small pregnancy cohort, showing several changes during pregnancy and/or after delivery.

Only a limited number of groups performed MALDI-MS analysis of IgA glycosylation before. These MALDI-TOF-MS experiments were performed in low resolution linear mode, and mass precision was not stated, or large mass deviations were allowed: ±1 Da (140-250 ppm; m/z window 3500-8000) for O-glycopeptide analysis, and 0.3% (3000 ppm) for sialidase treated N-glycopeptides. Of note, Tarelli et al. did not analyse tryptic peptides; therefore only the compositions can be compared to our data, and not the observed masses. Nevertheless, a large body of correctly annotated glycopeptide was thus gathered (Table 1; Supplementary Table S3). Generally, we were able to gain more information in the high mass region, whereas previous publications observed more low mass O-glycopeptides. We observed five O-glycopeptide compositions that have not been published previously. This may be caused by different sample preparation, for example a slight skewing of the spectra to higher masses because of the HILIC-SPE, or due to limited in-source fragmentation in our intermediate pressure FTICR mass spectrometer.

In addition, the use of an ultrahigh resolution MALDI-FTICR-MS circumvented LC steps - generally taking 30 to 120 min per sample, e.g. - prior to the MS measurement. For the MALDI approach a simple 2-step HILIC was necessary, taking only 60 minutes for 96 samples. The second step of the HILIC was necessary to obtain data concerning the Asn144 containing glycopeptide. However, this manual step can be automated to reduce hands-on time. Moreover, the intermediate pressure in the FTICR prevents the loss of sialic acids observed in MALDI-TOF-MS. Generally, the measurements and relative quantitation of IgA1 O-glycosylation in MALDI mode has been performed on de-sialylated glycopeptides, or on low-level sialylated myeloma species, thus losing the valuable information about the sialic acids. MALDI-MS analysis of the intact N-glycopeptides to obtain site-specific information has only been published once. Again, sialidase treated glycopeptides were analysed. The technique described in the current manuscript allows to analyse intact N- and O-glycopeptides bearing sialic acids in a high-throughput MALDI-MS approach. This allowed for the discovery of eight glycopeptides containing the Asn340 site, generally with higher sialylation compared to those reported in literature (Supplementary Table S3). Among these are some triantennary structures. Furthermore, a few non-fucosylated glycoforms were newly detected. Interestingly, the described technique without sialidase treatment allowed for the specific detection of non-sialylated species on Asn144 as well.
We observed the Asn340 containing glycopeptide with a truncated C-terminus, which is in accordance with previous reports. In our data the truncated version is more abundant than the non-truncated version, which is reflected in the higher spectral count in our proteomics analysis (42 vs. 16 counts). We assume that this truncation is naturally occurring on IgA from the circulation, and is not induced during storage or sample preparation. However, it is unclear which protease would be responsible for this processing, and whether it is of functional significance.

Both IgA subclasses (i.e. IgA1 and IgA2 isoforms) were captured in the here described procedure, as concluded from bottom-up protein identifications (Supplementary Table S1). However, IgA2 specific glycopeptides were not detected in our MALDI-FTICR-MS spectra. Likely, this is due to the rather low abundance of this subclass (~10%). Moreover, the expected masses of glycopeptides related to the IgA2 Asn205 containing tryptic peptide are outside the detection window.

In the current analysis of pregnancy-associated glycosylation changes on the glycopeptide level, we show changes in N-glycosylation and – to a lesser extent – O-glycosylation traits for IgA1. The site specific approach showed a decrease in sialylation after delivery (63% to 59%) for Asn144, whereas sialylation on Asn340 did not change. On the other hand, there was a pronounced increase in bisection on Asn340 from 1st trimester up to 6 weeks postpartum (52% to 58%), while the same process was observed on Asn144 to lower extent. Notably, our earlier pilot study by Ruhaak et al., which in contrast to the present study was performed merely on IgA-derived N-glycans, did not reveal pregnancy-associated glycosylation changes, which may be due to the fact that only samples from 6 individuals were analysed. Whether the changes are biologically important needs to be further investigated.

Surprisingly, IgA bisection shows a different time course than IgG bisection. While IgA bisection increases during pregnancy, and even further after delivery, IgG bisection remains at the same low level during pregnancy and only increases after delivery. After the first time point postpartum IgG bisection remains at the same level while IgA bisection decreases after this time point. The differences between IgA and IgG bisection time courses may have to do with their different biological roles and sites of action, yet this will require further research. In general, the role of bisection in modulating antibody function still needs to be clarified for both IgG and IgA.

For IgG-Fc N-glycans increased levels of galactosylation and sialylation during pregnancy compared to the non-pregnant state are well described in both healthy and diseased women. On IgG these changes are restricted to the Fc portion, whereas the Fab portion, carrying more processed glycans, does not exhibit these pregnancy associated changes in galactosylation and sialylation, although for the latter a trend is visible. Similarly, these changes are not observed for the diantennary glycans on Asn340 of IgA1, which are near completely processed with levels of galactosylation and sialylation of >99% and >89%, respectively.
Sialic acids on N-glycans of the standard serum IgA samples analysed in this study were almost exclusively α2,6-linked (>95%), which is in line with previous reports.\textsuperscript{10,25,45} With regard to the level of sialylation of N-glycans, Orczyk-Pawilowicz et al. reported no significant changes after delivery.\textsuperscript{25} However, in the current study we have found that sialylation levels at Asn144 were lower after delivery. These differences could be explained by the chosen time points: 6 weeks postpartum in our study versus 2 weeks after delivery in the earlier report. In addition, in this earlier study the glycosylation of IgA was detected by using lectins that are not site-specific. Differences in sialylation occurring at one site (e.g. 4% change at Asn144) can be obfuscated by the high levels (>89%) of sialylation on Asn340, as well as by the highly sialylated O-glycans.

In conclusion, we have developed a high-throughput method based on ultrahigh resolution MALDI-FTICR-MS that enables simultaneous profiling of IgA N- and O-glycosylation at the glycopeptide level. The ultrahigh resolving power allow for accurate detection of more than 50 O-glycopeptides and 6 and 13 glycoforms for the Asn144 and Asn340 N-glycopeptides, respectively. The suitability of the method to profile clinical samples was demonstrated by the application on a pregnancy cohort to study potential serum IgA glycosylation changes. In addition, this method can be used in conjunction with remodelling of IgA glycosylation and in vitro IgA bioactivity assays to study the functional relevance of IgA glycosylation with respect to antibody dependent cellular cytotoxicity and other effector mechanisms.

Methods

Human serum and plasma samples

Twenty-nine healthy Caucasian women who participated in the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study donated serum at the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} trimester of pregnancy and at six, twelve and >26 weeks postpartum.\textsuperscript{42} The PARA study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Centre, Rotterdam, The Netherlands, and experiments were performed accordingly. Informed consent was obtained from all subjects. In addition, EDTA plasma from a healthy donor was used as a technical control throughout the measurements.

Capture of polyclonal IgA

Human polyclonal IgA was captured from 10 μL human serum or plasma using CaptureSelect IgA beads. Twenty microliter bead slurry was applied to each well of a 96-well Orochem filter plate (10 μm pore size). The beads were pre-washed on a vacuum manifold with three times 200 μL PBS. Subsequently, the samples were diluted in 100 μL PBS on the plate, and incubated on a multiwell plate shaker for 1h. The beads with captured IgA were washed on the vacuum manifold with PBS and MQ water (3x 200 μL), followed by elution using 100 μL...
100 mM FA. The eluates were collected by centrifugation (1 min, 50×g) into V-bottom plates. Finally, the IgA samples were dried by vacuum centrifugation.

**Trypsin digestion**

The dried samples were reconstituted in 100 μL 20 mM ammonium bicarbonate buffer (pH 8) and reduced with 2 μL 125 mM DTT for 30 min at 60 °C. Sequentially alkylation with 2 μL 200 mM IAM was performed for 30 min at room temperature, followed by overnight digestion of IgA by TPCK treated trypsin at 37 °C, using 600 ng enzyme per well.

**HILIC enrichment and spotting**

Obtained trypsin digests of IgA samples were enriched for glycopeptides by a two-step microtip cotton HILIC SPE, using cotton thread as the solid phase, as described before, with minor modifications in the used solution volumes and composition. Briefly, 15 μL of the digest was transferred to a 96 wells V-bottom plate, and adjusted to 70% ACN by addition of 35 μL ACN. The cotton thread was washed three times with 20 μL MQ, and conditioned three times with 20 μL 70% ACN, using a 12-channel micropipette (2-20 μL). The sample was then loaded on the cotton by pipetting up-and-down 20 times. Subsequently, the cotton was washed 3 times with 20 μL 70% ACN containing 1% TFA and three washes with 20 μL 70% ACN, followed by elution in 5 μL MQ. Twenty-five microliter ACN was then added to the tryptic digest to bring the sample to 80% ACN for a sequential HILIC enrichment with 80% instead of 70% ACN, to capture glycopeptides that were not detectable in the 70% HILIC eluates. The eluates from both HILIC experiments were mixed separately with 15 μL 4-chloro-α-cyanocinnamic acid matrix (Cl-CCA; 0.33 mg/mL in acetone:ethanol 1:2) and 1 μL of each eluate was spotted on a Bruker AnchorChip plate with 800 μm anchors. All glycopeptide samples were spotted in duplicate, resulting in 384 spots per 96 serum samples.

**MALDI- and ESI-FTICR-MS**

Both MALDI- and ESI-FTICR-MS experiments were performed on a Bruker 15 Tesla solariX™ FTICR mass spectrometer equipped with a CombiSource (Bruker Daltonics, Bremen, Germany) and controlled by Compass solariXcontrol software. For details on the FTICR-MS settings see Supplementary Methods.

For profiling serum IgA1 N- and O-glycosylation, MALDI-FTICR-MS experiments were performed. Two duplicate spots were measured for each spotted sample. The FTICR system was externally calibrated using a dextran polysaccharide hydrolysate. The MALDI-FTICR-MS method introduced in this manuscript was applied to 174 serum samples from the cohort, 10 standard plasma samples from a healthy volunteer, and 12 blanks. All samples were spotted in duplicate and the whole workup was performed twice. In total 1536 MALDI spectra were obtained. DataAnalysis Software 4.0 SP4 (Bruker Daltonics) was used for the visualization and conversion of the MALDI spectra into .xy files.
For the characterization of some of the \(O\)-glycopeptides direct infusion ESI-FTICR-MS experiments were carried out using the settings specified in the Supplementary Methods section. Prior to MS/MS experiments the FTICR system was externally calibrated using a commercially available tune mix (Agilent).

**LC-ESI-MS/MS**

Additional structure confirmation of the \(N\)-glycopeptides was performed via nanoRP-LC-QTOF-MS/MS analysis on a high-resolution MaXis QTOF mass spectrometer (Bruker Daltonics) equipped with a captiveSpray nanoBooster source (Bruker Daltonics) coupled to a Ultimate 3000 nanoUPLC system (Dionex/Thermo Scientific, Breda, The Netherlands). The mass spectrometer and the LC were controlled by Hystar 3.2 (Dionex/Thermo Scientific) and data analysis was performed using DataAnalysis 4.2 (Bruker Daltonics). Experimental details on the LC-ESI-MS/MS method are specified in the Supplementary Methods section. MS/MS was performed using an inclusion list for the selected glycopeptides.

**Data processing**

The XY data of the MALDI-FTICR experiments were internally recalibrated with an in-house developed tool using a list of four \(N\)-glycopeptides (3\textsuperscript{rd} isotopic peak of Asn340 (truncated) bearing H5N4F1S1: \(m/z\) 4245.8192; Asn340 (truncated) H5N4F1S2: \(m/z\) 4536.9146; Asn340 (truncated) H5N5F1S2: \(m/z\) 4739.9940; Asn340 H5N5F1S2: \(m/z\) 4903.0579; oxidized +15.9949 Da) with and without oxidation, and seven \(O\)-glycopeptides (H4N4S2: \(m/z\) 6182.6181; H4N5S2: \(m/z\) 6385.6974; H4N4S3: \(m/z\) 6473.7135; H4N5S3: \(m/z\) 6676.7929; H4N5S4: \(m/z\) 6967.8883; H5N5S4: \(m/z\) 7129.9411; H5N5S5: \(m/z\) 7421.0365). The in-house developed tool sequentially integrated the data for all isotopic peaks that theoretically cover 99% of the analyte intensity, and determined the signal-to-noise ratios. In addition, a quality control (QC) value gave an indication of the quality of each analyte signal by analysing the deviation of the observed isotopic pattern from the theoretical pattern. The QC value calculation was adapted not to include noise in the equation.

**Generation of analyte list**

Although most literature depicted the \(N\)-glycans of IgA1 to be highly galactosylated and sialylated diantennary complex type, some also reported high mannone structures, as well as tri- and tetraantennary glycans. The \(O\)-glycans were found previously to be of various variants of the core 1 type. Therefore we started our analysis with a non-curated list of \(N\)- and \(O\)-glycopeptides. The list contained 65 potential \(N\)-glycoforms for each \(N\)-glycopeptide. Three \(N\)-glycopeptide sequences were included, namely LSLHRPALEDLLGSEANLTCTLGLR for Asn144, and LAGKPTHVNPSVMAEVDGTCY for Asn340, the latter with and without a truncated tyrosine (Y) at the C-terminus. Finally, for the Asn340 glycopeptides oxidation of the methionine (Met345) was included as a potential variable. In addition 182 potential \(O\)-glycopeptides (ranging from 1 HexNAc up to 6 HexNAc + 6 Hex + 8 SA) were included.
for extraction, considering a maximum of six occupied sites with core 1 O-glycans on the peptide HYTNPSQDVTPCPVPSTPSPSTPSPSTPSCCHPR. In total the list of potential analytes contained 507 (182+65+65+65+65+65) structures. The theoretical m/z values of the truncated Asn340 containing glycopeptide (both oxidized and non-oxidized) bearing a Man5 or H3N4 glycan were outside the measuring range and were therefore excluded. In order to curate the list of analytes the following steps were taken: analytes showing signal-to-noise greater than six in less than 50% of the spectra were removed from the analysis. In addition, the 25% quartile boundary of the QC value distribution had to be smaller than 0.03 for an analyte to be included in the final extraction. When two analytes were overlapping, the analyte with the lowest QC value was selected. In case of small differences in QC values, the expectancy based on the released N-glycan relative abundance was used to select the analyte. Finally, the internally calibrated spectra for the technical controls and for the healthy pregnant women were summed, resulting in two sum spectra for the analysis of the O- and the Asn340 N-glycopeptide clusters, and two for the Asn144 N-glycopeptide cluster. Again, the QC values for the analytes had to be < 0.03 for analytes to be included. Finally, the mass accuracy errors of the observed species were inspected to confirm the identity of the extracted species. A median error of less than 5 ppm was set as an inclusion threshold. In the final analyte list overlapping N-glycopeptides were curated based on the relative abundances of released glycans, which were obtained as described in the Supplementary Methods.

Quality threshold for MALDI-FTICR-MS spectra
In order to obtain high quality data, a curation step was performed on the obtained MALDI-FTICR-MS spectra. The total intensity per glycopeptide cluster was calculated based on the final analyte list. In addition, the percentage of glycopeptides with a signal-to-noise ratio (S/N) greater than six was calculated for each of the clusters. The total intensity was then plotted against the percentage with S/N > 6 (example in Supplementary Fig. S1). This plot was used as a guide to set cut-off values for both total intensity and the percentage. As a readout the average relative standard deviation per time point was monitored, as well as the percentage of spectra passing the cut-off threshold for which approximately 90% was considered as the lower limit. Finally, from each sample the highest intensity spectrum of the duplicates was selected.

Calculation of glycosylation traits
O-glycosylation features were calculated for each sample: the number of GalNAcs, galactoses, sialic acids, as well as the ratios for sialic acids per galactose and galactose per GalNAc. In addition, we checked the relative abundances of peptides with more GalNAcs than galactoses, indicative for the presence of Tn-antigen, and those with more sialic acids than galactoses. For all the N-glycopeptides sialylation and bisection of diantennary glycans was calculated. For the truncated Asn340 containing peptide additionally galactosylation
and fucosylation of diantennary glycans, and the abundance of triantennary glycans were calculated. The formulas used to calculate all the derived traits are shown in the Supplementary Methods.

**Statistical analysis**
The Wilcoxon signed-rank test was performed for paired samples without assumptions about normality. To correct for the three tests within each calculated glycosylation trait a Bonferroni correction for multiple testing was performed, with \( p < 0.017 \) considered as significant.

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**Supplementary Information**
Supplementary information is available online via http://www.nature.com/article-assets/npg/srep/2016/160615/srep27955/extref/srep27955-s1.pdf
References


CHAPTER 5

THE ASSOCIATION BETWEEN GALACTOSYLATION OF IMMUNOGLOBULIN G AND IMPROVEMENT OF RHEUMATOID ARTHRITIS DURING PREGNANCY IS INDEPENDENT OF SIALYLATION

Albert Bondt, Maurice H.J. Selman, André M. Deelder, Johanna M.W. Hazes, Sten P. Willemsen, Manfred Wuhrer, Radboud J.E.M. Dolhain

Abstract

Rheumatoid arthritis (RA) is known to improve during pregnancy and to flare after delivery. Changes in the glycosylation of immunoglobulin G (IgG)'s fragment crystallizable (Fc) have been suggested to play a role herein. Recent animal studies indicate that not galactosylation but mainly sialylation is important in this respect. We aim to find new associations between IgG-Fc N-glycosylation and improvement of RA during pregnancy and the flare after delivery. Sera of RA patients (n=251 pregnancies) and healthy controls (n=32), all participating in a prospective cohort study on RA and pregnancy (PARA study), were collected before conception, during pregnancy, and after delivery. Using a recently developed fast and robust nanoRP-HPLC-sheath-flow-ESI-MS method the glycosylation of IgG Fc-glycopeptides was measured in a subclass specific manner, with relative standard deviations of < 4% for the 8 most abundant IgG Fc glycopeptides during the entire measurement period of over 3 weeks. In patients and controls several glycosylation changes were observed during pregnancy. In depth analysis of the association of these glycosylation changes with disease activity revealed that galactosylation, independent of sialylation, is associated with improvement of RA during pregnancy. Functional studies in human cell systems should be performed to obtain more insight into this controversy.
Introduction

Autoimmune diseases provide a large economic threat in industrialized countries. Rheumatoid arthritis (RA) is one of these diseases, affecting 0.5 - 1% of the adult population, with women developing the disease three times more frequently than men. Interestingly, a spontaneous improvement of RA has been observed for many patients during pregnancy with a flare after delivery. Pregnancy is the only natural situation known to show this effect. Various immunomodulatory processes have been described during pregnancy in order to accommodate the fetus, e.g. (local) influx of T regulatory cells and regulation of cytokine and receptor expression, as reviewed by among others.

Antibodies are key players in the immune system, protecting the body from a large variety of threats. In autoimmune diseases, like RA, antibodies against self antigens, autoantibodies for short, are thought to represent an important pathogenic mechanism. The most abundant antibody in the human circulation is immunoglobulin G (IgG). IgG autoantibodies are present in patients with RA. IgGs are glycoproteins carrying predominantly biantennary complex-type N-glycans in the constant region of the heavy polypeptide chains of the fragment crystallizable (Fc) domain. This glycan contains a heptasaccharide core which may carry a core fucose, galactose(s), sialic acid(s) (SA) and/or a (bisecting) N-acetylgalcosamine (GlcNAc) (Figure 1A). IgG Fc glycosylation features strongly associate with the pro- and anti-inflammatory properties of IgG. However, a clear functional role for the IgG Fc glycosylation in determining disease activity in autoimmune diseases has not been demonstrated yet. It has been suggested that IgG Fc glycosylation changes play a role in pregnancy induced remission of RA. Pregnancy could, therefore, provide a good model system to study antibody related pathogenic mechanisms in RA and other autoimmune diseases, potentially revealing more in depth insight into the underlying mechanisms of autoimmunity.

Figure 1. A Schematic structure of a biantennary N-glycan of IgG Fc portions. The four human IgG subclasses show differences in the peptide sequence and a considerable variation in the structure of the attached N-glycan. B-D Mass spectra of a patient showing the triply charged IgG1 glycopeptides in the first trimester (B), the third trimester (C), or three months after delivery (D; masses depicted in Table S-2). Note the changes in ratios of G0F, G1F and G2F and the relative increase of G2FS during the third trimester. Disialylated species were detected only in trace amounts and were therefore not included in the calculations.
IgG Fc glycosylation can be studied by mass spectrometry (MS) at the level of intact proteins, glycopeptides and released glycans. While the high sample complexity of human polyclonal IgG complicates analysis at the intact glycoprotein level, (high throughput) analysis of glycopeptides or released glycans is relatively easy to perform. Both glycopeptide and released glycan analysis by MS provide detailed information on the glycoforms present. Glycopeptide analysis, however, is the only approach providing IgG subclass and site specific glycosylation information in a single analysis.

IgG subclass specific glycosylation analysis has frequently been performed by matrix assisted laser desorption (MALDI)-MS \(^ {16-18}\) and LC-MS.\(^ {19-21}\) While MALDI-MS is more potent in terms of analysis throughput, determination of IgG2 fucosylation and IgG4 glycosylation is hampered by the presence of isomeric glycopeptide species. By contrast, LC-MS analysis allows subclass specific IgG Fc glycosylation determination due to the additional separation prior to MS analysis. We recently developed a fast and highly robust nanoRP-HPLC-sheath-flow-ESI-MS method for the sensitive and subclass specific analysis of IgG Fc glycosylation where neutral and sialylated glycoforms of the same glycopeptide co-elute as a result of the trifluoroacetic acid (TFA) mobile phase additive.\(^ {21}\) The separation was coupled to the mass spectrometer by a sheath-flow ESI sprayer interface applying a sheath-liquid of isopropanol, propionic acid and water (50:20:30, v:v) to overcome TFA ion-suppression.

Here we applied this setup to analyze serum IgG Fc glycosylation profiles of 32 healthy pregnant Caucasian volunteers and 251 RA patient pregnancies at several time points before and during pregnancy, and after delivery. The very high robustness of the system allowed the analysis of over 1500 samples within four weeks with a RSD < 4% for the 8 most abundant IgG1 Fc glycopeptides. Various pregnancy associated glycosylation changes were observed in patients and controls. Interestingly, we observed an association with improvement of RA for IgG Fc galactosylation independent of sialylation, suggesting galactose could be the immunosuppressant sugar on IgG.

**Experimental procedures**

**Study population and data collection**

The current study is embedded in the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study, a nationwide prospective cohort study on pregnancy and RA. Two hundred and fifty-one pregnancies from 219 RA patients were included between 2002 and 2009.\(^ {2}\) One hundred twenty-one RA-patient pregnancies were followed from pre-conception, 101 from the first trimester of pregnancy, 16 from the second trimester and 13 from the third trimester onwards. Thirty-two patients participated twice. Only patients of whom at least three serum samples were available, of which at least one obtained during pregnancy and one postpartum, were included. All patients fulfill the 1987 ACR criteria for RA. As a reference group 32 healthy pregnant Caucasian volunteers without adverse obstetric history were followed from the first trimester onwards. Only completed pregnancies were included.
The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands.

**Categorization of disease activity and clinical response**

Disease activity was assessed using the DAS28 score based upon swollen and tender joint count and C-reactive protein (CRP). Improvement of RA was defined according to the European League Against Rheumatism (EULAR) response criteria (responders n=57; ‘non-responders’ n=64) (Table S-1 A). The response was defined between first and third trimester. The EULAR response criteria require an initial DAS28>3.2 at the first trimester (n=128) and could therefore not be applied to all patients. A postpartum flare was defined according to the so-called reversed EULAR response criteria (Table S-1 B). These reverse response criteria have no baseline requirement for DAS28. The flare was defined between 6 and 26 weeks postpartum (n=228).

**IgG sample preparation and measurement**

IgG glycosylation analysis was performed as described previously. Briefly, human polyclonal IgGs were captured from 2 µl of serum using Protein G Sepharose™ 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) in 96-well plate format. Captured IgGs were washed with PBS and water (3 x 200 µl), eluted with 100 mM formic acid (100 µl; Fluka, Steinheim, Germany) and dried by vacuum centrifugation. The purified IgGs (IgG1-4) were digested overnight at 37°C with 200 ng trypsin (Promega, Leiden, The Netherlands) in 25mM ammonium bicarbonate buffer (Fluka). Samples were centrifuged at 4000 rpm for 5 min and 250 nl aliquots were analyzed on a Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with a Dionex Acclaim PepMap100 C18 (5 µm particle size, 5 mm x 300 µm i.d.) trap column and an Ascentis Express C18 nano column (2.7 µm HALO fused core particles, 50 mm x 75 µm i.d.; Supelco, Bellefonte, USA). Separation was achieved in 16 minutes using a short gradient of 95% acetonitrile (Biosolve BV, Valkenswaard, the Netherlands;) and 0.1% trifluoroacetic acid (Fluka). The nano-reverse phase-HPLC separation was interfaced to a micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) by a sheath-flow-ESI sprayer (capillary electrophoresis ESI-MS sprayer; Agilent Technologies, Santa Clara, USA). To assist with the spray formation and reduce trifluoroacetic acid ion suppression a 2 µl/min sheath liquid was applied consisting of 20% propionic acid, 50% isopropanol and 30% water. Subclass specific (IgG1, IgG2/3 and IgG4) glycopeptide profiles were recorded from 300 to 2000 Dalton with 2 average scans at a frequency of 1 Hz. The collision energy and quadrupole ion energy of the MS were set to 4 and 2 eV, respectively. The Ultimate 3000 HPLC system and the Bruker micrOTOF-Q were operated by Chromeleon Client version 6.8 and micrOTOF control version 2.3 software, respectively.
Data analysis
Spectra were internally calibrated with a list of known glycopeptide masses using Bruker DataAnalysis 4.0 software in batch mode and exported to the open mzXML format. LC-MS data were aligned to a system standard using msalign2. Extraction of glycopeptide data was performed using Xtractor2D software. Both software tools were developed in-house and are freely available at www.ms-utils.org/Xtractor2D. Further analysis was performed using Microsoft Excel.

For 50 glycopeptide species (Table S-2; 20 IgG1 glycoforms, 10 IgG4 glycoforms, 20 IgG2/3 glycoforms) relative intensities were obtained by integrating and summing three isotopic peaks of the doubly and triply charged species followed by normalization to the total subclass specific glycopeptide intensities. Measurements with a combined glycopeptide signal intensity below 20000 counts were removed from the analysis. For measurements above this threshold a signal-to-noise ratio higher than 3:1 was used as an additional inclusion criterion.

Glycan species calculations
On the basis of the normalized intensities the percentage of biantennary N-glycans carrying galactose(s), a core fucose, a sialic acid and a bisecting N-acetylglucosamine were calculated. The following formulae were used, where the abbreviations depict the number of galactoses (G0, G1, G2), fucose (F), bisecting GlcNAc (N), and sialic acid (S):

\[
\text{Galactosylation} = (G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S + G1NS)*0.5 + G2F + G2FN + G2FS + G2FNS + G2 + G2N + G2S + G2NS for IgG1 and IgG2/3, and (G1F + G1FN + G1FS + G1FNS)*0.5 + G2F + G2FN + G2FS + G2FNS for IgG4.
\]

\[
\text{Sialylation} = G1FS + G1FNS + G1S + G1NS + G2FS + G2FNS + G2S + G2NS for IgG1 and IgG2/3, and G1FS + G1FNS + G2FS + G2FNS for IgG4.
\]

\[
\text{Bisecting GlcNAc} = G0FN + G0N + G1FN + G1FNS + G1N + G1NS + G2FN + G2FNS + G2N + G2NS for IgG1 and IgG2/3, and G0FN + G1FN + G1FNS + G2FN + G2FNS for IgG4.
\]

\[
\text{Fucosylation} = G0F + G0FN + G0FN + G0 + G0N for IgG1 and IgG2/3, and G0F + G0FN for IgG4.
\]

To separate the effects of galactosylation and sialylation we calculated the abundances of non-galactosylated (Gal-0), monogalactosylated (Gal-1), digalactosylated (Gal-2), sialylated Gal-2 and non-sialylated Gal-2 glycoforms, and determined the ratio ‘sialylated Gal-2’:‘Gal-2’. The following formulae were used:

\[
\text{Gal-0} = G0F + G0FN + G0 + G0N for IgG1 and IgG2/3, and G0F + G0FN for IgG4.
\]

\[
\text{Gal-1} = G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S + G1NS for IgG1 and IgG2/3, and G1F + G1FN + G1FS + G1FNS for IgG4.
\]

\[
\]
Ratio sialylated species in Gal-2 = (G2FS + G2FNS + G2S + G2NS)/Gal-2 for IgG1 and IgG2/3, and (G2FS + G2FNS)/Gal-2 for IgG4. See Figure 1 for a detailed representation of the glycan structures and mass spectra.

**Statistical analysis**

To study glycosylation changes in time, and differences between patients and their healthy references, longitudinal analysis was performed using the MIXED Procedure in SAS/STAT® 9.2 (SAS Institute Inc., NC, USA). The response variable was the level of glycosylation (IgG1 galactosylation, sialylation, fucosylation or presence of bisecting GlcNAc; IgG2/3 galactosylation, sialylation, fucosylation or presence of bisecting GlcNAc; IgG4 galactosylation, sialylation, or presence of bisecting GlcNAc) and the covariate was the interaction of time (as a categorical variable) and group (patient or healthy). To model the correlation we used a random intercept for each patient and assumed that the residual correlation was autoregressive of order one.

To investigate which covariates are associated with the levels of galactosylation, sialylation, fucosylation and incidence of bisecting GlcNAc a mixed model was estimated. The following covariates were tested: time point, use of prednisone, methotrexate or biologicals, DAS28, rheumatoid factor (RF) positivity and anti-citrullinated protein antibody (ACPA) positivity, and maternal age at delivery. All covariates were entered simultaneously into the model. In these multivariate models the within-patient correlation was modeled as described above. Additional statistical analyses were performed using Stata/SE 12.0 for Windows (StataCorp LP, TX, USA): changes in galactosylation, sialylation, fucosylation and incidence of bisecting GlcNAc were calculated as the value at the third trimester minus the value at first trimester for the change during pregnancy, and as the value at 26 weeks postpartum minus the value at six weeks postpartum for the change after delivery. Two-group mean-comparison tests were performed to identify possible differences between responders and non-responders (during pregnancy), or ‘flare’ and ‘no flare’ (after delivery).

The association between disease activity and galactosylation or sialylation was established by linear regression analysis. Disease activity was used as the dependent variable, while Gal-0, Gal-1, Gal-2, sialylated Gal-2, non-sialylated Gal-2 and the degree of sialylation of Gal-2 were used as independent variables in separate analyses. In addition a regression analysis was performed with disease activity as dependent variable and the degree of galactosylation and sialylation put first separately (univariate) into the model, followed by a multivariate analysis in which both variables were put simultaneously into the model.

For all analyses a two-sided *p*-value < 0.05 was considered to be significant.
To evaluate IgG Fc glycosylation changes with pregnancy and RA, tryptic IgG glycopeptides of 251 RA and 32 healthy pregnancies (Table 1) were prepared and analyzed using a recently described nanoRP-HPLC-sheath-flow-ESI-MS method. The use of disease modifying anti-rheumatic drugs (DMARDs) in this cohort has been described in detail and is comparable to the current study. Unambiguous assignment of 50 glycoforms was allowed on the basis of literature knowledge of IgG N-glycosylation.

The short acetonitrile gradient combined with the fused core particle column resulted in baseline separation of the different human IgG subclasses with glycopeptides of IgG1 eluting first followed by IgG4 and IgG2/3. Accurate relative quantification for each of the assigned IgG Fc N-glycopeptides was achieved by integrating and summing multiple isotopic peaks of all observed charge states. To allow for batch-to-batch corrections a plasma standard was included in triplicate on each 96-well sample plate. Furthermore, every 32nd run a glycopeptide standard was injected to evaluate

### Table 1. Cohort Characteristics

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<th>Patient pregnancies (n=251)</th>
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<tr>
<td>Mean age at delivery in years (SD)</td>
<td>32.8 (3.8)</td>
<td>32.1 (4.4)</td>
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<tr>
<td>Median disease duration at delivery in years (range)</td>
<td>5.6 (0.7 to 29.7)</td>
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<tr>
<td>Number of nulliparous women, n (%)</td>
<td>129/251 (51.4)</td>
<td>14/32 (43.8)</td>
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<tr>
<td>Mean gestational age at delivery, weeks (range)</td>
<td>39.2 (30.4 to 42.3)</td>
<td>40.1 (34.7 to 42.0)</td>
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</tr>
<tr>
<td>ACPA+ positive, n (%)</td>
<td>135/227 (59.5)</td>
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<td></td>
</tr>
<tr>
<td>Rheumatoid Factor (IgM) positive, n (%)</td>
<td>162/248 (65.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Erosive disease, n (%)</td>
<td>151/248 (60.8)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DAS28-3(CRP) &gt;3.2 in first trimester, n (%)</td>
<td>128/218 (58.7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Response during pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>good response/moderate response, n (%)</td>
<td>57/121 (47.1)</td>
<td>-</td>
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</tr>
<tr>
<td>no response, n (%)</td>
<td>64/121 (52.9)</td>
<td>-</td>
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<tr>
<td>Flare during postpartum period (flare)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>severe deterioration/moderate deterioration, n (%)</td>
<td>70/228 (30.7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>no deterioration, n (%)</td>
<td>158/228 (69.3)</td>
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<tr>
<td>Median number of DMARDs (incl prednisone and biologicals) prior to conceive (min-max)</td>
<td>2 (0-7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Use of biologicals prior to conceive, n (%)</td>
<td>30/251 (12.0)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>No DMARD use prior to conceive, n (%)</td>
<td>39/251 (15.5)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Use of methotrexate prior to conceive, n (%)</td>
<td>144/251 (57.4)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

ǂ Abbreviations: ACPA – anti-citrullinated peptide antibodies; DAS-28(3)-CRP – disease activity score based on three variables including CRP; DMARD – disease modifying anti-rheumatic drugs

### Results

**IgG glycosylation measurement and robustness**

To evaluate IgG Fc glycosylation changes with pregnancy and RA, tryptic IgG glycopeptides of 251 RA and 32 healthy pregnancies (Table 1) were prepared and analyzed using a recently described nanoRP-HPLC-sheath-flow-ESI-MS method. The use of disease modifying anti-rheumatic drugs (DMARDs) in this cohort has been described in detail and is comparable to the current study. Unambiguous assignment of 50 glycoforms was allowed on the basis of literature knowledge of IgG N-glycosylation. The short acetonitrile gradient combined with the fused core particle column resulted in baseline separation of the different human IgG subclasses with glycopeptides of IgG1 eluting first followed by IgG4 and IgG2/3. Accurate relative quantification for each of the assigned IgG Fc N-glycopeptides was achieved by integrating and summing multiple isotopic peaks of all observed charge states. To allow for batch-to-batch corrections a plasma standard was included in triplicate on each 96-well sample plate. Furthermore, every 32nd run a glycopeptide standard was injected to evaluate
the system performance. Interestingly, no batch effects were observed and the variability remained below 4.0% for IgG1 (based on 8 most abundant glycopeptides representing 95% of the total detected IgG1 glycoforms), below 2.9% for IgG2/3 (7 most abundant species, 96%), and below 3.8% for IgG4 (6 most abundant species, 94%) during the entire measurement period of over 3 weeks for both standards (Figure S-1).

**Table 1**

<table>
<thead>
<tr>
<th>Cohort Characteristics</th>
<th>Patient pregnancies</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at delivery in years</td>
<td>32.8 (3.8)</td>
<td>32.1 (4.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Median disease duration in years</td>
<td>5.6 (0.7 to 29.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of nulliparous women</td>
<td>129/251 (51.4)</td>
<td>14/32 (43.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Mean gestational age at delivery</td>
<td>39.2 (30.4 to 42.3)</td>
<td>40.1 (34.7 to 42.0)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>135/227 (59.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatoid Factor (IgM) positive, n (%)</td>
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<td></td>
</tr>
<tr>
<td>DAS28-3(CRP) &gt;3.2 in first trimester, n (%)</td>
<td>128/218 (58.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response during pregnancy</td>
<td>Good response/moderate response, n (%)</td>
<td>57/121 (47.1)</td>
<td></td>
</tr>
<tr>
<td>Flare during postpartum period</td>
<td>Severe deterioration/moderate deterioration, n (%)</td>
<td>70/228 (30.7)</td>
<td></td>
</tr>
<tr>
<td>Median number of DMARDs (incl prednisone and biologicals) prior to conceive (min - max)</td>
<td>2 (0 - 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of biologicals prior to conceive, n (%)</td>
<td>30/251 (12.0)</td>
<td></td>
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<tr>
<td>No DMARD use prior to conceive, n (%)</td>
<td>39/251 (15.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of methotrexate prior to conceive, n (%)</td>
<td>144/251 (57.4)</td>
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**Figure 2.** IgG1 Fc-glycosylation changes during pregnancy and after delivery. Galactosylation (A; p<0.0001), sialylation (B; p<0.0001), incidence of bisecting GlcNAc (C) and fucosylation (D; p<0.01) are given for RA patients (black) and healthy controls (gray). Error bars represent the 95% confidence intervals. Abbreviations: GlcNAc, N-acetylglucosamine; trim, trimester of pregnancy; wk, weeks; pp, postpartum.
IgG glycosylation changes during pregnancy and postpartum

In RA patients galactosylation increased for IgG1 from 54.3% (standard deviation (SD) 9.7%) preconception to 59.3% (SD 8.3%) in the first trimester and 64.9% (SD 7.8%) in the third trimester (p<0.0001). After delivery the level of IgG1 galactosylation dropped to 55.0% (SD 8.0%) twenty-six weeks postpartum (p<0.0001; Figure 2A). A similar change was obtained for IgG2/3 and IgG4 (Table S-3). Levels of galactosylation were higher in controls than in cases (p<0.005 for all subclasses; Table S-3).

Next to changes in galactosylation also sialylation, bisection and fucosylation were analyzed. IgG1 sialylation increased for IgG1 in cases from 16.0% (SD 3.8%) before conception to 18.7% (SD 4.1%) in the first trimester of pregnancy up to 21.4% (SD 4.3%) in the third trimester (p<0.0001). A marked decrease was observed after delivery, reaching the lowest levels at 12 weeks postpartum (16.1%; SD 3.5%; p<0.0001; Figure 2B). Similar results were obtained for IgG2/3 and IgG4 (Table S-3). Sialylation levels were higher in controls than in cases (p<0.01 for IgG1 at all time points and IgG4 during pregnancy; Table S-3).

The incidence of bisecting GlcNAc on IgG1 in patients decreased from 13.7% (SD 2.7%) before pregnancy to 12.3% (SD 2.5%) in the second trimester of pregnancy (p<0.0001). ‘Recovery’ was found to start in the third trimester of pregnancy and at 26 weeks after delivery a maximum of 14.2% (SD 2.9%) was reached (p<0.0001; Figure 2C). Similar results were obtained for IgG2/3 and IgG4 (Table S-3). Incidence of bisecting GlcNAc did not differ significantly between RA patients and controls.

IgG1 fucosylation decreased in RA patients from 94.6% (SD 2.8%) before conception to 93.7% (SD 2.8%) in the third trimester (p<0.0001). An increase was observed to 94.3% (SD 2.5%) at 12 weeks after delivery (p<0.0001). For controls also an increase was observed between the third trimester of pregnancy (92.0%; SD 3.1%) and 26 weeks after delivery (92.9%; SD 2.8%; p<0.0001; Figure 2D). Similar changes were observed for IgG2/3 (Table S-3). For IgG1 higher levels of fucosylation were observed in cases compared to controls (p<0.05) (Table S-3).

Changes in glycosylation are more pronounced in responders

Patients were classified according to the change in disease activity score during pregnancy in accordance with the EULAR response criteria as responders (n=57) and non-responders (n=64). Significantly different changes were observed for IgG1 galactosylation of responders (7.4% increase; SD 4.6%) versus non-responders (4.7% increase; SD 4.4%; p<0.01). Similar results were obtained for IgG2/3 and IgG4 (Figure 3A). Changes of the level of IgG1 sialylation were significantly different for responders (3.2% increase; SD 2.2%) versus non-responders (2.3% increase; SD 2.0%; p<0.05). Trends could be observed for IgG2/3 and IgG4 (Figure 3B). No such differences were observed for fucosylation and incidence of bisecting GlcNAc.

Changes in IgG glycosylation are more pronounced in postpartum flaring patients

Cases with a flare in disease activity between 6 and 26 weeks postpartum, classified in accordance with the so-called reverse EULAR response criteria, showed a more pronounced
decrease in galactosylation of IgG1 (-4.0%; SD 5.1%) than cases with no flare (-1.8%; SD 4.2%; p<0.01), and of IgG2/3 and IgG4 (Figure 3C). For sialylation significant differences were observed on IgG1 (flare: -1.7% (SD 1.8%) versus no flare: -0.9% (SD 2.0%); p<0.01), and IgG4, whereas a trend could be observed for IgG2/3 (Figure 3D).

No significant differences were observed for fucosylation and incidence of bisecting GlcNAc.

Figure 3. Patients who improve during pregnancy (responders) show a more pronounced increase in galactosylation (A) and sialylation (B) compared to non-responders during that period. After delivery a decrease in galactosylation (C) and sialylation (D) is more pronounced in patients for whom a flare of disease activity is observed compared to those without a flare. ** p≤0.01; * p<0.05

Multivariate analysis to identify covariates that associate with levels of glycosylation

To investigate which covariates are associated with levels of glycosylation a mixed model was estimated in which all covariates were entered simultaneously. Fc galactosylation was negatively associated with disease activity (all subclasses; p<0.01) and prednisone use (IgG1 and IgG2/3; p<0.05). The same was observed for sialylation (all subclasses; p<0.01, and IgG1 and IgG2/3; p<0.05, respectively).

Fucosylation levels were lower with a higher age at delivery (p<0.05). For IgG1, fucosylation was found to be increased with higher disease activity (p<0.0001). The incidence of bisecting GlcNAc of all subclasses was increased with higher age at delivery (p<0.05). For all subclasses negative associations were observed for bisecting GlcNAc and prednisone use (p<0.05). In addition, for IgG1 negative associations were found for bisecting GlcNAc with the use of biologicals (p<0.05) and ACPA positivity (p<0.01), and for IgG2/3 incidence of bisecting GlcNAc with ACPA positivity (p<0.001) and disease activity (p<0.01).
**Galactosylation is associated with disease activity irrespective of sialylation**

Next, we studied the effects of galactosylation and sialylation on DAS28 in more detail. Regression analysis of DAS28 and IgG1 Fc galactosylation revealed a negative association ($R^2=0.110; \beta=-0.33; p<0.0001$). Likewise, DAS28 showed a negative association with Fc sialylation ($R^2=0.051; \beta=-0.23; p<0.0001$). When a linear regression model with both galactosylation and sialylation as independent variables was built the $R^2$ was only slightly increased ($R^2=0.130; p<0.0001$) as compared to the model using galactosylation alone. Notably, in this combined model only galactosylation showed a negative association with disease activity ($\beta=-0.61$), while the association of sialylation with disease activity was found to be positive ($\beta=0.31$).

To analyze these phenomena in more detail, the association between disease activity and agalactosylated (no galactose, no sialic acid; Gal-0; Figure 4A), monogalactosylated (one galactose, irrespective of sialic acid; Gal-1; Figure 4B), or digalactosylated forms (two galactoses, irrespective of sialic acid; Gal-2; Figure 4C) was determined. This analysis clearly showed that a lower percentage of Gal-0, and a higher percentage of Gal-2 is associated with lower disease activity ($p<0.0001$), which is in line with literature. To determine whether the effect of increased galactosylation on disease activity is mediated through sialylation, the presence of Gal-2 glycoforms was subdivided into (mono)sialylated and non-sialylated glycans (please note that only trace amounts of disialylated Gal-2 were detected using our mass spectrometric approach). Interestingly, this analysis showed that the negative association of Gal-2 with disease activity occurred irrespective of sialylation. When these associations were studied in more detail, the directional coefficient of non-sialylated Gal-2 was found to be more negative, and the effect size ($R^2$) larger than for sialylated Gal-2 (Figure 4D and E; $p<0.0001$). In order to further dissect the differences observed between sialylated and non-sialylated Gal-2, we analyzed the association between the percentage of sialylated Gal-2 glycoforms within the total Gal-2 fraction and disease activity (Figure 4F) which was found to be positive ($p<0.0001$). Thus, while digalactosylation is associated with low disease activity (Figure 4C, D), our data indicate that an increased level of sialylation of the digalactosylated species is associated with higher disease activity (Figure 4F).
Here we applied a fast and robust nanoRP-HPLC-sheath-flow-ESI-MS method to study pregnancy and RA associated IgG Fc glycosylation changes. Most reversed phase (RP) LC separations of IgG Fc glycopeptides apply an acetonitrile (ACN) gradient with formic acid (FA) as acidic mobile phase additive, providing the possibility to couple the separation directly to the MS. However, using FA sialylated glycopeptides show higher retention than neutral glycopeptides of the same IgG subclass as a result of secondary interactions with the stationary phase. By replacing FA with TFA these secondary interactions were effectively suppressed resulting in similar retention for neutral and acidic glycopeptides of the same IgG subclass. As a result of this co-elution and the constant and LC gradient.

**Discussion**

Figure 4. Galactosylation is strongly associated with disease activity. The association of disease activity with agalactosylated, monogalactosylated and digalactosylated IgG1 glycoforms is shown (A-C; p<0.0001, p<0.06, p<0.0001 respectively). To determine the effect of sialylation on the association between disease activity and digalactosylation (C), this association was determined separately for non-sialylated IgG1 Gal-2 (D) and mono-sialylated IgG1 Gal-2 (E), showing in both cases a negative association with disease activity. Note the larger R2 for the non-sialylated species (D) compared to sialylated species (E). This is further illustrated in F, which demonstrates a minor but positive association of IgG Gal-2 sialylation with disease activity (p<0.0001). Abbreviations: DAS28-3(CRP) = disease activity score based on three variables including CRP; Gal-0 = N-glycan carrying no galactose; Gal-1 = N-glycan carrying one galactose; Gal-2 = N-glycan carrying two galactoses.
dependent spray obtained with the sheath-flow sprayer, neutral and sialylated glycoforms experience similar ionization effects which improves the accuracy of the IgG subclass specific relative quantification. The nanoRP-HPLC-sheath-flow-ESI-MS setup proved to be extremely robust as can be derived from the very low RSDs (< 4% for the 8 most abundant IgG Fc glycopeptides) obtained for the IgG Fc glycopeptide standard, which was measured more than 50 times during the entire measurement time of 3 weeks.

We found that various glycosylation changes occur during pregnancy. Changes in galactosylation and sialylation are particularly pronounced in responders during pregnancy, and in patients with a flare in disease activity after delivery. For fucosylation and incidence of bisecting GlcNAc the associations with disease activity were less pronounced and were only present for certain IgG subclasses. Furthermore, this study shows that the association between disease activity of RA and the glycosylation of IgG is mainly dependent upon galactosylation and not upon sialylation.

Galactosylation of IgG has been shown to be associated with the improvement of RA during pregnancy, as well as with disease outcome. An important role for the galactosylation of IgG in determining the outcome of the immune response has been demonstrated in mice: arthritis could be induced with lower concentrations of anti-collagen antibodies when the galactoses were enzymatically removed from the administered IgG.

Recent studies, however, questioned the central role of galactose in determining the function of IgG. In both an arthritis and immunothrombocytopenia (ITP) mouse model it was shown that the removal of sialic acids inhibited the immunosuppressive effect of intravenous immunoglobulins (IVIG) to levels similar to those observed after removal of the entire N-glycan, suggesting that the immunosuppressive effect is mainly sialic acid dependent. Therefore, it was hypothesized that the previously described associations of galactosylation with disease state had to be attributed to sialylation, since the N-glycan galactoses serve as a carrier for sialic acid. However, this central role for sialic acid in IVIG-induced immune suppression in inflammatory disease mouse models was not confirmed by others.

This controversy prompted us to determine whether either the galactosylation or sialylation increase is associated with lower disease activity in RA. Our study revealed that the observed association between galactosylation and patient disease activity is not due to sialic acids, since the association of the galactosylation of the N-glycan with disease activity was independent of the sialylation (Figure 4D). In fact we observed that increased sialylation was associated with higher levels of disease activity (Figure 4F), indicating that sialic acid opposes the association of galactosylation. Previous studies on the specific function of galactosylation in relation to sialylation in humans reported non-conclusive results or were unable to detect sialic acid as accurately as in the present study due to the chosen analytical approach.

Since our data suggest that galactosylation but not sialylation is associated with disease activity in RA, it is important to speculate on how increased galactosylation could result in lower RA disease activity. A role for mannose binding lectin has been suggested herein, but
this could not be confirmed in later studies. Recently dectin-1 gained attention with respect to the immunosuppressive role of IgGs. As demonstrated in a mouse model, immune complexes containing highly galactosylated IgGs promoted the association of Fc-gamma-RIIb and dectin-1 on neutrophils which leads to an inhibition of the proinflammatory effector functions of complement protein C5a. One may speculate whether highly galactosylated IgG induces immune suppression in pregnancy and RA via a similar mechanism. In line with previous reports, fucosylation was found to be increased in patients compared to controls. Moreover, increased fucosylation was found to be associated with higher disease activity. This is somewhat unexpected as literature points to an anti-inflammatory role of increased fucosylation.

The results of this study are in line with a potential immunomodulatory role of IgG glycans in the improvement of RA during pregnancy, but mechanistic studies would obviously be needed to prove such a causative role of IgG glycosylation changes in the suppression of autoimmunity in RA. Getting a better understanding of the function of certain glycans on IgG will allow a more rational design of monoclonal antibodies, when used as therapeutics or diagnostics in patient care.

In conclusion, this study underlines the importance of N-glycans on the Fc-portion of antibodies, and their possible role in inflammation and immune suppression. By studying the naturally occurring glycosylation changes in relation to RA disease activity during pregnancy, we have been able to determine associations between glycosylation and inflammation. Our data imply possible anti-inflammatory properties of galactosylation, but not of sialylation. Together with changes in fucosylation and bisecting GlcNAc during pregnancy, a complex multifactorial system is suggested, in which galactosylation is a major player, whereas other glycan features seem to fine tune the inflammatory response.

Acknowledgment
This project is funded by the Dutch Arthritis Foundation (NR 10-1-411) and by the European Union’s Seventh Framework Program (FP7-Health-F5-2011) under grant agreement n°278535 (HighGlycan). Maurice H. J. Selman thanks Hoffmann la Roche for financial support.

Associated content
Supporting Information. EULAR response criteria; reversed EULAR response criteria; IgG1, IgG2/3 and IgG4 glycopeptide masses; variation in most abundant glycopeptides of standard sample; numerical representation observed glycosylation features controls and cases at each timepoint; statistics glycosylation changes over time. This material is available free of charge via the Internet at http://pubs.acs.org/doi/suppl/10.1021/pr400589m.
References


Fab glycosylation of immunoglobulin G does not associate with improvement of rheumatoid arthritis during pregnancy

Albert Bondt, Manfred Wuhrer, T. Martijn Kuijper, Johanna M.W. Hazes, Radboud J.E.M. Dolhain

Abstract

Background: Changes in immunoglobulin G (IgG) constant domain (Fc) glycosylation are associated with changes in rheumatoid arthritis (RA) disease activity in response to pregnancy. Here, we sought to determine whether the same holds true for variable domain (Fab) glycosylation.

Methods: IgGs were captured from RA and control sera obtained before (RA only), during and after pregnancy, followed by Fc and Fab separation, glycan release, and mass spectrometric detection. In parallel, glycans from intact IgG were analyzed. The data was used to calculate glycosylation traits, and to estimate the level of Fab glycosylation.

Results: The overall level of Fab glycosylation was increased in RA patients compared to controls, while no differences in Fab glycosylation patterns were found. For the Fc and intact IgG (Total) previously observed differences in galactosylation and bisection were confirmed. Furthermore, increased galactosylation of Fc and Total were associated with lower disease activity and autoantibody positivity. In addition, the change in Fc galactosylation associated with the change in disease activity during pregnancy and after delivery, while this was not the case for Fab.

Conclusions: In contrast to changes in Fc glycosylation, changes in Fab glycosylation are not associated with improvement of RA during pregnancy and arthritis flare after delivery.
**Background**

Rheumatoid arthritis (RA) is an autoimmune disease, for which it is well known that patients may improve during pregnancy.\(^1\) The IgG fragment crystallisable (Fc) N-glycan compositions, specifically the levels of galactosylation and sialylation (Figure 1), have been recognised to be different in RA-patients compared to healthy controls and to be associated with RA disease activity and its improvement during pregnancy.\(^2\)-\(^4\)

In addition to the Fc moiety, that always bears N-galactose, the hypervariable region of the antigen binding fragment (Fab) may harbour N-galactose.\(^5\) These glycans are generally present on approximately 15-25% of the Fab portions, and have been found to alter the binding properties of antibodies to their antigen, as well as effector functions, and are known to change during pregnancy.\(^6\)-\(^10\) Interestingly, for rheumatoid arthritis specific autoantibodies (anti-citrullinated protein antibodies (ACPA)) the vast majority has been shown to carry these Fab glycans.\(^11\)

In view of the known differences between the Fc N-glycans of RA patients and healthy controls, and its association with disease activity and improvement during pregnancy, we aimed to determine whether similar changes and associations can be found regarding the Fab N-glycans. In addition, the level of Fab glycosylation was investigated.

**Figure 1. Schematic representation of Immunoglobulin G.** Indicated is what is generally considered as the Fc and Fab or F(ab’)2 portion. The glycans of both Fc and Fab consist of the same building blocks, although the most abundant glycans may differ vastly in their appearance.

This figure was originally published in Bondt et al., Immunoglobulin G (IgG) Fab Glycosylation Analysis Using a New Mass Spectrometric High-throughput Profiling Method Reveals Pregnancy-associated Changes. *Mol Cell Proteomics.* 2014; 13:3029-3039. © the American Society for Biochemistry and Molecular Biology, and slightly adapted for the current manuscript.
Methods

Study population and data collection

For the current study we used sera from the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study, a prospective cohort study on pregnancy and RA. At all time points disease activity (DAS28; based upon swollen and tender joints and CRP) and medication was recorded. For the analysis we selected a subset of RA-patients (n=33) with the most pronounced (>=0.6) or no change in disease activity, both during and after pregnancy, in search of maximum contrast. Only sera obtained before pregnancy, during the third trimester of pregnancy, and 26 weeks postpartum were analyzed. In addition sera obtained from 32 healthy pregnant volunteers were analyzed (third trimester and 6 months after delivery only). All patients fulfill the 1987 ACR criteria for RA. The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands.

IgG capturing and N-glycan release

IgGs were affinity-captured in duplicate from human serum in a 96-wells format, as described before, with minor modifications. One set was used for IdeS digestion to separate the Fab and Fc portions, whereas the other set was kept intact to analyze the total IgG glycans. Dried samples were reconstituted in 5µL phosphate buffered saline and 10µL 2% (w/v) sodium dodecyl sulfate solution, instead of 10 and 20, respectively. Similarly, PNGaseF was added in 10µL.

Sialic acid derivatization and HILIC enrichment

To prevent the loss of sialic acids in the mass spectrometric detection of released glycans a derivatization protocol was applied as described before, with minor modifications. Briefly, 2 µL released glycans were added to 20 µL ethyl esterification reagent in a V-bottom 96-wells plate, and incubated at 37 °C for 1h. After the incubation 20 µL acetonitrile was added. Ethyl esterified N-glycans were purified from the reaction mixture using hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE) with a piece of cotton thread as the solid phase in a micro tip as described before.

MALDI-TOF-MS and data processing

Five microliter of eluate was mixed on spot with sodium hydroxide spiked Super-DHB matrix (Sigma) on an AnchorChip plate. Automated matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) measurements were performed summing 10000 shots per spot with 250 shot steps and a full spot random walk. Details on data processing, extraction, and the calculation of glycosylation traits are described in Additional file 1.
Statistical analysis
Statistical analysis was performed in Stata 13SE. Bonferroni correction for multiple testing was performed throughout, with final significance thresholds depicted in the tables with results. First, a Wilcoxon rank-sum test was performed to explore the differences in glycosylation between RA patients and healthy controls outside pregnancy, at ≥26 weeks after delivery. Next we investigated which clinical covariates (use of medication, autoantibody (AAb) positivity, age at delivery and disease activity) were associated with the glycosylation in RA patients using linear regression analysis. To study changes in glycosylation over time the Wilcoxon matched-pairs signed-rank test was used, comparing pre-conception with the third trimester for the patients, and third trimester with 26 weeks postpartum for both patients and controls. Association of the changes in glycosylation over time and the accompanying changes in disease activity were explored using Spearman correlation.

Results
Response during pregnancy and flare after delivery
Clinical characteristics of the patients and controls are given in Additional file 1: Table S1. The patients that improved (n=14) during pregnancy showed a mean decrease in DAS28 (delta DAS28; ΔDAS) of -1.7 (SD 0.5), whereas the nine patients that worsened showed a ΔDAS of +1.6 (SD 0.5). Ten patients did not change during pregnancy (ΔDAS=0.0; SD 0.4). After delivery the twelve patients that flared had a mean ΔDAS of +1.5 (SD 0.8), while the twelve improving patients showed a ΔDAS of -1.5 (SD 0.6). Nine patients without a change in disease activity between the third trimester and six months postpartum (ΔDAS=-0.1; SD 0.4) were selected.

Fab glycosylation is higher in RA patients outside pregnancy when compared to controls, yet with similar glycans
The calculated level of Fab glycosylation in the patients (21.4%; [IQR 19.5-25.4%]) was significantly higher than in controls (16.5%; [IQR 13.3-18.2%]) at the non-pregnant time point 26 weeks postpartum (Tables 1, 2 and 3). However, no differences between the levels of galactosylation, sialylation, fucosylation or presence of bisecting N-acetylglucosamine were observed for the Fab glycosylation (Tables 1, 2 and 3) compared between healthy controls and RA patients. However, for both Fc and total IgG (Total) there was a lower level of galactosylation and higher level of bisection in RA patients compared to the healthy controls.

Galactosylation of Fc and intact IgG, but not Fab, associate with disease activity and autoantibody positivity
In addition, it was explored which clinical covariates were associated with the levels of the calculated glycosylation traits at 26 weeks postpartum. Covariates were first tested univariate, and those with p<0.2 were included in the multivariate analysis. No Fab
glycosylation models remained significant after Bonferroni correction. However, for galactosylation of both Fc and Total multivariate models could be composed with $R^2$-values of 0.4 and 0.5, respectively. For these models the main contributing factors were disease activity (beta=-0.4) and autoantibody positivity (beta=+0.5). The results for all multivariate models are shown in Additional file 1: Table S2.

Table 1. Median percentages and corresponding interquartile ranges for all time points and glycosylation traits

<table>
<thead>
<tr>
<th></th>
<th>Healthy 3rd trim</th>
<th>26 wkpp</th>
<th>RA pre-conception</th>
<th>RA 3rd trim</th>
<th>26 wkpp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (%)</td>
<td>IQR</td>
<td>Median (%)</td>
<td>IQR</td>
<td>Median (%)</td>
</tr>
<tr>
<td>Galactosylation Fc</td>
<td>81.5 [78.1-83.2]</td>
<td>71.6 [67.9-74.7]</td>
<td>60.8 [52.2-64.3]</td>
<td>73.6 [66.5-79.5]</td>
<td>61.0 [56.8-66.7]</td>
</tr>
<tr>
<td>Fucosylation Fc</td>
<td>96.8 [95.8-97.4]</td>
<td>97.3 [96.6-98.0]</td>
<td>97.9 [97.5-98.3]</td>
<td>97.5 [96.5-98.0]</td>
<td>97.7 [97.2-98.2]</td>
</tr>
<tr>
<td>Galactosylation Total</td>
<td>82.9 [80.6-84.6]</td>
<td>74.8 [70.3-77.0]</td>
<td>66.0 [59.9-69.0]</td>
<td>77.5 [73.5-82.2]</td>
<td>68.6 [59.1-73.5]</td>
</tr>
<tr>
<td>Fucosylation Total</td>
<td>95.4 [94.7-96.2]</td>
<td>96.5 [95.5-97.2]</td>
<td>96.9 [96.2-97.3]</td>
<td>96.2 [95.4-96.7]</td>
<td>96.7 [96.0-97.1]</td>
</tr>
<tr>
<td>Galactosylation Fab</td>
<td>96.8 [96.3-97.0]</td>
<td>97.2 [96.5-97.8]</td>
<td>96.6 [95.7-97.7]</td>
<td>97.3 [96.9-97.8]</td>
<td>96.8 [96.0-97.3]</td>
</tr>
<tr>
<td>Sialylation Fab</td>
<td>81.6 [80.4-84.3]</td>
<td>79.0 [77.8-82.6]</td>
<td>79.7 [77.0-81.1]</td>
<td>82.7 [80.6-84.6]</td>
<td>80.2 [76.9-81.6]</td>
</tr>
<tr>
<td>Fucosylation Fab</td>
<td>70.6 [68.0-75.3]</td>
<td>76.6 [74.5-80.6]</td>
<td>75.6 [71.9-80.5]</td>
<td>73.2 [61.6-75.8]</td>
<td>77.7 [72.8-79.7]</td>
</tr>
<tr>
<td>Bisection Fab</td>
<td>37.0 [36.1-41.4]</td>
<td>46.6 [41.7-52.2]</td>
<td>51.5 [46.5-55.2]</td>
<td>42.1 [33.4-44.4]</td>
<td>52.2 [46.7-54.6]</td>
</tr>
</tbody>
</table>

*Abbreviations used: trim = trimester; wkpp = weeks postpartum; IQR = interquartile range
Table 2. Number of observations obtained for each category and time point

<table>
<thead>
<tr>
<th></th>
<th>Healthy 3rd trim*</th>
<th>Healthy 26 wkpp</th>
<th>RA 3rd trim</th>
<th>RA 26 wkpp</th>
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<tbody>
<tr>
<td>Fc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactosylation</td>
<td>29 / 25</td>
<td>33 / 31</td>
<td>33 / 33</td>
<td></td>
</tr>
<tr>
<td>Sialylation</td>
<td>29 / 25</td>
<td>33 / 31</td>
<td>33 / 33</td>
<td></td>
</tr>
<tr>
<td>Fucosylation</td>
<td>29 / 25</td>
<td>33 / 31</td>
<td>33 / 33</td>
<td></td>
</tr>
<tr>
<td>Bissection</td>
<td>29 / 25</td>
<td>33 / 31</td>
<td>33 / 33</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25 / 25</td>
<td>33 / 30</td>
<td>31 / 31</td>
<td></td>
</tr>
<tr>
<td>Fc</td>
<td>17 / 15</td>
<td>29 / 25</td>
<td>23 / 25</td>
<td></td>
</tr>
<tr>
<td>Galactosylation</td>
<td>17 / 15</td>
<td>29 / 25</td>
<td>23 / 25</td>
<td></td>
</tr>
<tr>
<td>Sialylation</td>
<td>17 / 15</td>
<td>29 / 25</td>
<td>23 / 25</td>
<td></td>
</tr>
<tr>
<td>Fucosylation</td>
<td>17 / 15</td>
<td>29 / 25</td>
<td>23 / 25</td>
<td></td>
</tr>
<tr>
<td>Bissection</td>
<td>17 / 15</td>
<td>29 / 24</td>
<td>21 / 21</td>
<td></td>
</tr>
<tr>
<td>% glycosylation</td>
<td>15 / 15</td>
<td>29 / 24</td>
<td>21 / 21</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations used: trim = trimester; wkpp = weeks postpartum; pre-conc. = pre-conception.

Table 3. p-values obtained for comparing glycosylation traits of controls vs. cases and over time. For the comparison of RA vs. healthy a significance threshold of p<0.0038 was used (Bonferroni corrected for 13 tests), and for the time comparisons p<0.0013 (corrected for 42 tests). Significant observations are highlighted in bold font.

<table>
<thead>
<tr>
<th></th>
<th>RA vs. healthy at 26 wkpp*</th>
<th>Healthy 3rd trim vs. 26 wkpp</th>
<th>RA 3rd trim vs. 3rd trim</th>
<th>RA 3rd trim vs. 26 wkpp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactosylation</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Sialylation</td>
<td>0.077</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
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<tr>
<td>Fucosylation</td>
<td>0.072</td>
<td><strong>0.000</strong></td>
<td>0.003</td>
<td>0.048</td>
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<tr>
<td>Bissection</td>
<td>0.004</td>
<td>0.003</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Total</td>
<td><strong>0.001</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Total</td>
<td>0.179</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
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<tr>
<td>Fucosylation</td>
<td>0.889</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td>0.012</td>
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<tr>
<td>Bissection</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Fab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactosylation</td>
<td>0.347</td>
<td>0.333</td>
<td>0.004</td>
<td>0.148</td>
</tr>
<tr>
<td>Sialylation</td>
<td>0.754</td>
<td>0.028</td>
<td><strong>0.000</strong></td>
<td>0.007</td>
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<tr>
<td>Fucosylation</td>
<td>0.709</td>
<td>0.005</td>
<td><strong>0.000</strong></td>
<td>0.003</td>
</tr>
<tr>
<td>Bissection</td>
<td>0.104</td>
<td>0.005</td>
<td><strong>0.000</strong></td>
<td><strong>0.001</strong></td>
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<tr>
<td>% glycosylation</td>
<td><strong>0.002</strong></td>
<td>0.401</td>
<td>0.009</td>
<td>0.249</td>
</tr>
</tbody>
</table>

*Abbreviations used: wkpp = weeks postpartum; trim = trimester
**Fab glycosylation changes during pregnancy and after delivery are not associated with changes in disease activity**

Galactosylation and sialylation of Fab, Fc and Total increased between pre-conception and the third trimester of pregnancy in RA patients (Tables 1, 2 and 3; Figure 2). The opposite was observed after delivery in both patients and healthy controls, except for the Fab portion where only a decrease in sialylation was observed. For fucosylation and bisection a decrease during pregnancy (RA only) and increase after delivery was observed for Fab, Fc and Total, in both patients and controls (Tables 1, 2 and 3). In addition, during pregnancy an increase in the level of Fab glycosylation was found in RA patients. Of note, changes in glycosylation traits that were high, e.g. Fab galactosylation and Fc fucosylation, were often not significant after Bonferroni correction (Tables 1, 2 and 3; Figure 2).

The Fc galactosylation change from pre-conception to the third trimester of pregnancy was negatively associated with the change in disease activity in that time span ($\rho=-0.39$, $p=0.029$; Additional file 1: Table S3; Additional file 1: Figure S1). The opposite was observed for the change Fc and Total galactosylation from third trimester to 26 weeks postpartum ($\rho=0.48$ and 0.62, $p=0.006$ and 0.001, respectively). For neither the Fab glycosylation traits nor the Fab glycosylation level these associations were observed.

![Figure 2](image_url)

**Figure 2.** Fc and Fab galactosylation and sialylation in RA and healthy controls. The percentages of galactosylation (yellow circle) and sialylation (purple diamond) on the Fc show a clear increase during pregnancy (from pre-conception to the 3rd trimester; RA only) and decrease after delivery (from 3rd trim to 26 weeks postpartum), as well as differences between cases and controls. No differences between cases and controls, and only minor pregnancy-associate changes were observed for the Fab glycosylation. Abbreviations used: pre-conc = pre-conception; trim = trimester; wkpp = weeks postpartum
Discussion

There is a strong association of IgG Fc galactosylation with RA disease activity and the pregnancy-associated improvement thereof.²,³ For the Fab portion of IgG this has not yet been investigated. Therefore, we applied our recently developed method for the analysis of Fab glycosylation on samples from a set of RA and healthy individuals in the context of pregnancy. Interestingly, we found no qualitative differences in Fab glycosylation between cases and controls nor an association with changes in disease activity when we compared several glycosylation traits. This may in part be caused by the fact that Fab galactosylation and sialylation levels are already high in the non-pregnant state, leaving less room for a potential pregnancy-associated increase.

Since the Fab portion of IgG – in contrast to the Fc portion – is not always glycosylated, the association of changes in Fab glycosylation with disease activity could be quantitative rather than qualitative. In line with this, it has recently been published that the RA specific ACPA autoantibodies express high levels of Fab glycosylation.¹¹ Indeed, in this study we did observe increased levels of Fab glycosylation. However, even though the levels are higher in RA patients compared to controls, they were not associated with disease activity.

In the current study we chose to include only a limited number of patients and time points. Therefore minor associations between changes in RA disease activity and changes in Fab glycosylation could have been missed.

Conclusions

In conclusion, this study clearly demonstrates that, in contrast to changes in the glycosylation of the IgG Fc domain, changes in the glycosylation of the IgG Fab domain do not seem to play a major role in the pregnancy associated improvement of RA.

Ethics approval and consent to participate

The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands. All patients gave informed consent.

Acknowledgements

The authors thank Mahmoud Habeeb for his efforts in testing purification and measurement conditions.

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Availability of data and materials
Additional files are available online:
MediaObjects/13075_2016_1172_MOESM1_ESM.docx
MediaObjects/13075_2016_1172_MOESM2_ESM.xlsx

Additional Files
Additional file 1: Additional Methods, Figure and Tables. The additional file contains an
addition to the Methods, a figure, and three additional tables. (DOCX)
Additional file 2: Data used for analysis. Contains the relative abundances of the observed
glycans and the calculated glycosylation traits for the RA patients and healthy controls used
in the current study. (XLSX)
References


CHAPTER 7

IgA N- AND O-GLYCOSYLATION PROFILING REVEALS NO ASSOCIATION WITH THE PREGNANCY-RELATED IMPROVEMENT IN RHEUMATOID ARTHRITIS

Albert Bondt, Simone Nicolardi, Bas C. Jansen, T. Martijn Kuijper, Johanna M.W. Hazes, Yuri E.M. van de Burgt, Manfred Wuhrer, Radboud J.E.M. Dolhain

Submitted manuscript.
Abstract

Objectives: The Fc glycosylation of immunoglobulin (Ig) G is well known to associate with rheumatoid arthritis (RA) disease activity. The same may be true for other classes of immunoglobulins. In the current study we sought to determine whether the glycosylation of IgA was different between healthy and RA, and whether it associated with RA disease activity, in particular with the pregnancy-associated improvement thereof or the flare after delivery.

Methods: A recently developed high-throughput method for glycoprofiling of IgA1 was applied to affinity captured IgA from sera of RA cases (n=252) and healthy controls (n=32) collected before, during and after pregnancy.

Results: IgA1 O-glycans were shown to bear more sialic acids in RA compared to controls. In addition, levels of bisecting N-acetylgalactosamine of the N-glycans at asparagine 144 were higher in the patients. The levels of several N-glycosylation traits were shown to change with pregnancy, similar to what has been shown before for IgG. However, the changes in IgA glycosylation were not associated with improvement or a flare of disease activity.

Conclusions: The glycosylation of immunoglobulin A differs between RA patients and healthy controls. However, our data suggest only a minor, if any, association of IgA glycosylation with RA disease activity.
Introduction

In rheumatoid arthritis (RA) autoantibodies, such as rheumatoid factor (RF) and anticitrullinated peptide antibodies (ACPA), are thought to be crucial not only in initiating the disease process of RA, but also in the more chronic stages.\(^1\)\(^-\)\(^4\) Both for RF as well as ACPA representatives of the immunoglobulins (Ig) G, A and M have been recognized.\(^2\)\(^,\)\(^3\)\(^,\)\(^5\) Besides autoantibodies, hormones are thought to have an important role in the pathogenesis of RA, as can be illustrated by the spontaneous improvement of RA during pregnancy.\(^6\)\(^-\)\(^8\)

Antibodies are glycoproteins, meaning that they bear at least one glycan on the protein backbone.\(^9\) Glycans may influence immunological properties of antibodies by affecting for example receptor binding, half-life, or binding of complement.\(^10\)\(^,\)\(^11\) There are two main classes of glycans described for antibodies, namely the N-linked glycans (linked to a nitrogen atom, or \(N\)) and the O-linked glycans (linked to an oxygen atom, or \(O\)). The N-glycans can be found at the asparagine in a known consensus sequence, Asn-Xxx-Ser/Thr, where Xxx can be any amino acid except proline, while O-linked glycans can be linked to serine or threonine residues, generally in a proline rich region.

During pregnancy N-glycosylation changes have been described for several individual serum proteins, for example alpha-1-antitrypsin, alpha-1-acid glycoprotein, IgG, and IgA, as well as for released total serum N-glycans.\(^12\)\(^-\)\(^15\) For IgG, bearing almost exclusively N-linked glycans, these changes during pregnancy and after delivery were shown to be associated with RA disease activity, and the pregnancy-associated improvement thereof.\(^8\)\(^,\)\(^16\) For the glycosylation of the other immunoglobulins little is known concerning the association with RA and the pregnancy-associated improvement of RA disease activity. However, given the evident role of antibodies in RA this information has a high relevance.

Indications for a potential pathogenic role of IgA in RA are given by studies demonstrating the association of IgA rheumatoid factor autoantibodies with bone erosions in RA.\(^17\)\(^,\)\(^18\) Similarly, RA patients that are seropositive for both IgG- as well as IgA-ACPA have a more severe disease course compared to patients with only IgG-ACPA.\(^19\)

There are two subclasses of IgA, of which IgA1 is the most abundant (approximately 90%) in the human circulation. While most immunoglobulin isotypes carry only N-linked glycans, the IgA1 subclass carries in addition between three and six O-linked glycans.\(^20\)\(^,\)\(^21\) The O-glycans are present in the hinge region of IgA1, and consist of one N-acetylgalactosamine (GalNAc), which can be decorated by a galactose (Gal; Figure 1). Furthermore, sialic acids (SA) may be attached to either the galactose or the GalNAc. There are two N-glycosylation sites on IgA1 (Asn144 and Asn340), and the glycans are of the diantennary complex type, generally consisting of a core of four N-acetylgalactosamines (GlcNAcs), three mannoses and two galactoses. This core structure can be decorated by one or two sialic acids, one fucose, or one bisecting GlcNAc (Figure 1).\(^12\)\(^,\)\(^15\)\(^,\)\(^22\)\(^,\)\(^24\) The addition of the fucose has been shown to occur only at Asn340.\(^23\) In addition, low amounts of triantennary glycans can be detected at this site.
Disease associated IgA O-glycosylation changes have been demonstrated in several IgA related diseases, like IgA nephropathy, Henoch-Schönlein purpura, Wiskott-Aldrich syndrome and X-linked thrombocytopenia, for which decreased levels of galactose on the O-glycans were observed.\textsuperscript{25-27} For RA the published data are less clear, mainly due to small sample size and to the applied techniques with only a low level of detail. Up to now it has been suggested that in RA (n=26) the number of galactoses on the O-glycans is similar to controls, while the level of GalNAc is decreased.\textsuperscript{28} The level of GalNAc on the IgA O-glycans was found to be associated with the level of galactosylation on the N-glycans of IgG. With regards to the IgA1 N-glycosylation in RA no differences were found in comparison to healthy individuals (n=5 for both groups).\textsuperscript{22}

![Figure 1. Schematic representation of IgA and IgG and the sites of their respective glycosylation sites as can be detected by glycopeptide analysis of serum derived samples. Insets show schematic representations of an O-glycan, an N-glycan, and a potential configuration of an O-glycopeptide. No linkage information is intended by any position of a monosaccharide.](image)

However, to study the relevance of IgA glycosylation in association with RA disease activity and pregnancy-induced improvement thereof, larger numbers of samples need to be analysed in detail, as we have previously reported for IgG.\textsuperscript{8} For this purpose we recently developed a high-throughput technique for the site-specific analysis of both IgA N- as well as O-glycosylation.\textsuperscript{15} By applying this method we have been able to show pregnancy-associated changes both in N- and O-glycosylation of IgA in a cohort of healthy women. In the current manuscript we apply this method on the RA-samples collected within the framework of the PARA (Pregnancy-associated Amelioration of Rheumatoid Arthritis) study. In total the used sample set consists of approximately 1600 samples obtained from pre-pregnancy and onwards from both healthy controls and RA patients. These samples allow to study
differences between healthy controls and RA-patients, the association of IgA glycosylation with disease activity in the non-pregnant state, as well as for research on the improvement during pregnancy and flare after delivery and its association with IgA-glycosylation.

Patients and methods

Study population
The current research is embedded in the PARA study, a nationwide prospective cohort study on pregnancy and RA (n=253) from pre-pregnancy and onwards. Healthy controls (n=32) were included as a reference group. The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands. This study has been described in detail.

Categorisation of disease activity and clinical response
Disease activity was assessed using the DAS28 score based upon C-reactive protein (CRP), and swollen and tender joint count. Responders and non-responders were categorized based upon the EULAR response criteria. The response was defined between first and third trimester. A postpartum flare was defined according to the so-called reversed EULAR response criteria. The flare was defined between 6 and 26 weeks postpartum.

IgA sample preparation and measurement
Site-specific IgA glycosylation analysis was performed as described before. Briefly, IgA was captured from 10 µL human serum or plasma using CaptureSelect IgA beads (Life Technologies Europe, Bleiswijk, The Netherlands) in 96-wells format. After elution from the beads the samples were dried, followed by overnight digestion at 37 °C with TPCK treated trypsin (Sigma Aldrich, Steinheim, Germany) after reduction/alkylation. Obtained trypsin digests of IgA samples were enriched for glycopeptides by a two-step microtip cotton HILIC SPE, using cotton thread as the solid phase, as described before. Matrix-assisted laser desorption/ionization (MALDI)- Fourier transform ion cyclotron resonance (FTICR)- mass spectrometry (MS) measurements were performed as described before.

Data extraction and curation
DataAnalysis Software 4.0 SP4 (Bruker Daltonics) was used for the visualization and conversion of the MALDI spectra into .xy files. The XY data of the MALDI-FTICR experiments were internally recalibrated and integrated with an in-house developed tool (MassyTools v1.6.3.0). In addition, several quality measures were extracted. After analyte and spectrum curation, several glycosylation traits were calculated as described in the Supplementary Methods.
Statistical methods

Initial data exploration using SIMCA showed minor batch effects, which were corrected using the ComBat batch correction in R. All other statistical tests were performed using Stata/SE 13.1 for Windows (StataCorp LP, TX, USA). In the batch corrected data first the differences between RA patients and healthy controls were investigated in the non-pregnant state at 6 months postpartum using a Mann-Whitney test. To study which covariates were associated with the levels of the extracted N- and O-glycosylation traits of RA patients outside pregnancy, multivariable linear regression analysis was performed on the samples obtained at 26 weeks postpartum. Covariates that were studied were the use of prednisone, methotrexate, sulfasalazine, hydroxychloroquine, leflunomide, or TNF-inhibitors (all different TNF-inhibitors grouped together), the DAS28, autoantibody (RF, ACPA, or both) seropositivity, and age at delivery.

The effects of pregnancy on the glycosylation traits was studied using multilevel mixed-effects linear regression, similar to what has been described before, but with the Stata software. Comparisons were made between: pre-conception and the third trimester (RA only), first and third trimester, third trimester and six weeks postpartum, third trimester and 26 weeks postpartum, and between six and 26 weeks postpartum (bisection only). Furthermore, it was studied whether there were differences in the pregnancy-associated glycosylation changes between patients which do or do not improve during pregnancy (responders and non-responders), as well as between patient with or without a postpartum flare of disease activity, as described before.

Finally, we explored which glycosylation traits are associated with disease activity at each time point, using a bootstrap approach for a multivariable linear regression model. The pre-conception time point was excluded for a lack of power. The disease activity score was used as the dependent variable, whereas the use of prednisone, methotrexate, sulfasalazine, hydroxychloroquine, biologicals, or leflunomide, autoantibody (RF, ACPA, or both) seropositivity, age at delivery as fixed elements, and the calculated glycosylation traits (for IgA as obtained in the current study, and for IgG obtained in a previous study) were selected as potential covariates. A thousand rounds of backwards elimination with p<0.05 cut-off were performed, after which a summary of all models was prepared using the ten most often included glycosylation variables.

In all statistical tests Bonferroni multiple testing correction was performed when applicable, as specified in the presented tables.

Results

Study population

Clinical characteristics of patients and healthy controls are presented in Table 1.
### Table 1. Cohort characteristics

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<th>Healthy n=32</th>
<th>RA patient pregnancies n=252</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at delivery in years (SD)</td>
<td>32.1 (4.4)</td>
<td>32.8 (3.7)</td>
</tr>
<tr>
<td>Median disease duration in years at first visit (range)</td>
<td>4.9 (0.2-28.6)</td>
<td>4.9 (0.2-28.6)</td>
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<td>ACPA positive patients, n (%)</td>
<td>153/252 (61)</td>
<td>153/252 (61)</td>
</tr>
<tr>
<td>RF positive patients, n (%)</td>
<td>161/239 (67)</td>
<td>161/239 (67)</td>
</tr>
<tr>
<td>ACPA and/or RF positive patients, n (%)</td>
<td>181/252 (72)</td>
<td>181/252 (72)</td>
</tr>
<tr>
<td>Erosive disease, n (%)</td>
<td>150/246 (61)</td>
<td>150/246 (61)</td>
</tr>
<tr>
<td>Response during pregnancy(^1), n (%)</td>
<td>56/120 (47)</td>
<td>56/120 (47)</td>
</tr>
<tr>
<td>Flare during postpartum period, n (%)</td>
<td>69/223 (31)</td>
<td>69/223 (31)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Per time point</th>
<th>Pre-conception</th>
<th>1(^{st}) trimester</th>
<th>2(^{nd}) trimester</th>
<th>3(^{rd}) trimester</th>
<th>6 weeks postpartum</th>
<th>12 weeks postpartum</th>
<th>26 weeks postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease activity score, mean (SD)</td>
<td>3.6 (1.1)</td>
<td>3.6 (1.1)</td>
<td>3.6 (1.1)</td>
<td>3.3 (1.1)</td>
<td>3.3 (1.1)</td>
<td>3.6 (1.2)</td>
<td>3.4 (1.1)</td>
</tr>
<tr>
<td>Use of prednisone, n (%)</td>
<td>37/121 (31)</td>
<td>79/223 (35)</td>
<td>86/234 (37)</td>
<td>81/239 (34)</td>
<td>84/240 (35)</td>
<td>87/242 (36)</td>
<td>78/242 (32)</td>
</tr>
<tr>
<td>Use of sulfasalazine, n (%)</td>
<td>41/121 (34)</td>
<td>62/223 (28)</td>
<td>64/234 (27)</td>
<td>61/239 (26)</td>
<td>60/240 (25)</td>
<td>72/242 (30)</td>
<td>70/242 (29)</td>
</tr>
<tr>
<td>Use of hydroxychloroquine, n (%)</td>
<td>9/121 (7)</td>
<td>5/223 (2)</td>
<td>5/234 (2)</td>
<td>4/239 (2)</td>
<td>9/240 (4)</td>
<td>18/242 (7)</td>
<td>17/242 (7)</td>
</tr>
<tr>
<td>Use of methotrexate, n (%)</td>
<td>0/121 (0)</td>
<td>0/223 (0)</td>
<td>0/234 (0)</td>
<td>0/239 (0)</td>
<td>34/240 (14)</td>
<td>59/242 (24)</td>
<td>74/242 (31)</td>
</tr>
<tr>
<td>Use of leflunomide, n (%)</td>
<td>0/121 (0)</td>
<td>0/223 (0)</td>
<td>0/234 (0)</td>
<td>0/239 (0)</td>
<td>0/240 (0)</td>
<td>3/242 (1)</td>
<td>4/242 (2)</td>
</tr>
<tr>
<td>Use of TNF-inhibitors, n (%)</td>
<td>5/121 (4)</td>
<td>0/223 (0)</td>
<td>0/234 (0)</td>
<td>0/239 (0)</td>
<td>13/240 (5)</td>
<td>23/242 (10)</td>
<td>29/242 (12)</td>
</tr>
</tbody>
</table>

\(^1\) The EULAR response criteria require a DAS28>3.2 at baseline.
IgA glycosylation in RA patients and healthy controls outside pregnancy

Site-specific differences in glycosylation between patients and controls

The difference between RA patients and healthy individuals was tested at ≥ 6 months postpartum, when the women had ‘recovered’ from pregnancy. For the majority of the calculated glycosylation traits no difference was observed between patients and controls (Table 2). However, the number of sialic acids on the O-glycans was significantly higher in RA patients (3.1; standard deviation (SD) 0.18) compared to healthy controls (3.0; SD 0.14; \( p = 0.0023 \)). For the N-glycosylation at Asn144 the level of bisecting GlcNAc was higher in patients (32.0%; SD 8.1%) compared to the controls (26.3%; SD 4.1%; \( p = 0.0001 \); Table 2).

Table 2. Glycosylation comparison of healthy controls vs. RA patients at the non-pregnant state (≥ 26 weeks after delivery. Bonferroni corrected \( p < 0.004 \) is considered significant, marked in bold font.

<table>
<thead>
<tr>
<th>Glycosylation</th>
<th>Healthy</th>
<th>RA</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( O )-glycosylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># GalNAc</td>
<td>4.81</td>
<td>0.009</td>
<td>4.82</td>
</tr>
<tr>
<td># Gal</td>
<td>3.99</td>
<td>0.014</td>
<td>4.03</td>
</tr>
<tr>
<td># SA</td>
<td>3.05</td>
<td>0.027</td>
<td>3.15</td>
</tr>
<tr>
<td>SA per Gal</td>
<td>0.76</td>
<td>0.006</td>
<td>0.78</td>
</tr>
<tr>
<td>Gal per GalNAc</td>
<td>0.83</td>
<td>0.003</td>
<td>0.84</td>
</tr>
<tr>
<td>( N )-glycosylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% sialylation</td>
<td>60.37</td>
<td>0.854</td>
<td>58.09</td>
</tr>
<tr>
<td>% bisection</td>
<td>26.27</td>
<td>0.784</td>
<td>32.04</td>
</tr>
<tr>
<td>Asn340</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intact % sialylation</td>
<td>95.68</td>
<td>0.170</td>
<td>95.17</td>
</tr>
<tr>
<td>trunc. % sialylation</td>
<td>89.82</td>
<td>0.173</td>
<td>90.13</td>
</tr>
<tr>
<td>intact % bisection</td>
<td>57.94</td>
<td>1.057</td>
<td>60.45</td>
</tr>
<tr>
<td>trunc. % bisection</td>
<td>56.12</td>
<td>0.893</td>
<td>57.26</td>
</tr>
<tr>
<td>trunc. % fucosylation</td>
<td>93.57</td>
<td>0.304</td>
<td>92.98</td>
</tr>
<tr>
<td>trunc. % triantennary</td>
<td>5.30</td>
<td>0.298</td>
<td>4.97</td>
</tr>
</tbody>
</table>

Association of IgA glycosylation with clinical variables

To determine which clinical parameters were associated with IgA glycosylation in RA patients outside pregnancy (at six months postpartum) multivariable linear regression analyses were performed, using the glycosylation traits as dependent, and several clinical parameters as independent variables. No models remained significant after Bonferroni correction \( (p < 0.0039) \). The best model was for bisection of the N-glycans at Asn340 (trunc.; \( p = 0.0042 \), \( R^2 = 0.12 \)), suggesting positive associations of disease activity \( (\beta = 0.21) \), use of prednisone \( (\beta = 0.21) \) and age at delivery \( (\beta = 0.16) \); Supplementary Table S1) with bisection.
Pregnancy-associated IgA glycosylation changes

Pregnancy-associated changes were studied using multilevel mixed-effects linear regression models. For the healthy controls the results were highly similar to what we published before,\textsuperscript{15} with only minor differences in significance due to a different statistical method and \( p \)-value cut-off. For the RA patients all \( N \)- and \( O \)-glycosylation variables, except for sialylation at the intact Asn340, showed pregnancy-associated changes (Supplementary Table S2). The \( O \)-glycans showed an increase in levels of GalNAc during pregnancy, and increased levels of Gal, SA, as well as increased ratios of SA per Gal and Gal per GalNAc after delivery. All changes were minor (0.3-2\% increase). At \( N \)-glycosylation site Asn144 an increase (1.04-fold) of sialylation was observed during pregnancy, and a decrease (0.96-fold) after delivery. The bisection at this site showed an increase during pregnancy (1.05 up to 1.09-fold), ongoing until six weeks postpartum. At Asn340 similar trends were observed, although the effect size for sialylation was 10-fold smaller, whereas the pregnancy-associated changes in bisection appear to be slightly more pronounced. Fucosylation, which is not present at Asn144, showed a slight decrease with pregnancy (0.99-fold), and increase after delivery (1.01-fold). The most pronounced effect sizes were observed for the low levels triantennary glycans at Asn340 which increased 1.10-fold during pregnancy and showed a 0.90- down to 0.86-fold decrease postpartum. For all glycosylation traits the observed effect sizes were highly similar between RA patients and healthy controls.

For the non-truncated Asn340 sialylation, and for Asn144 and truncated Asn340 fucosylation, the mixed model showed no deviating time points after Bonferroni correction (Supplementary Table S2). The means for each time point of all traits are depicted in Supplementary Table S3.

IgA glycosylation changes are not different between responders and non-responders

To study whether the changes in IgA glycosylation associate with the improvement of the RA disease activity during pregnancy (the response) and the worsening after delivery Wilcoxon rank-sum tests were performed for each calculated glycosylation trait. No significant differences were identified.

Furthermore, we explored the association of IgA as well as IgG glycosylation traits with disease activity. In the vast majority of the models IgG glycosylation traits were found to be associated with the disease activity. Only at the second and third trimester of pregnancy a minor influence of IgA glycosylation was observed. More details of the results are described in the Supplementary material.

Discussion

This study shows that IgA glycosylation is different between RA patients compared to healthy controls, and that pregnancy-associated changes in IgA glycosylation traits occur in the patients in a similar fashion as compared to the controls. However, these changes were not associated with the pregnancy-induced improvement of RA.
Several differences in IgA N- and O-glycosylation between RA patients and healthy controls were observed. However, these changes only showed minor effect sizes. Previously it was shown for the IgA O-glycosylation that the number of GalNACs is lower in RA patients. However, this was not confirmed in our study. The most likely explanation lies in the fact that we have been able to observe more high mass glycopeptides without losing the lower mass region, causing the differences in the number of GalNACs (which would be observed mainly in the lower mass glycopeptides) to lose significance. In contrast, we do show an increased level of sialic acids on the O-glycans in RA patients (1.03-fold), which would be visible mainly in the high mass region.

In the current study we observed hardly any changes with regards to the O-glycosylation during pregnancy. However, for the N-glycosylation we have been able to show that in RA patients several pregnancy-associated IgA N-glycosylation changes do occur, similar to what we have previously described for healthy controls. In addition, IgA N-glycosylation appears to show pregnancy-associated changes which are comparable to those observed for IgG-Fc, albeit at different levels. Furthermore, differences between RA patients and healthy controls were observed, with lower levels of N-glycan sialylation at Asn144, and increased levels of bisecting GlcNAc on the N-glycans at both Asn144 and Asn340. The higher level of bisection has been shown to associate with a less favourable state in several diseases, both on IgG and on cell surface glycans. Interestingly, the pregnancy-associated time lapse for IgA bisection was slightly different from what was observed previously for IgG, with an increase from the first trimester onwards until the first time point postpartum, only after which a decrease starts. For IgG the levels of bisection were relatively stable during pregnancy, and similarly stable (albeit at a higher level) after delivery. Thus, although the general pattern of pregnancy induced changes in glycosylation of serum IgA and IgG – both produced predominantly by plasma cells – appears to be similar, the functional consequences might be different for IgA compared to IgG and remain to be elucidated.

Finally, we sought to gain more insight into the potential association of IgA glycosylation with RA disease activity. The observed pregnancy-associated changes in glycosylation did not differ between patients that improved during pregnancy and patients that did not improve, nor between patients with and without a postpartum flare of disease activity. Furthermore, we explored this association using multivariable linear regression analysis, using the disease activity as the dependent variable. Models were built with a bootstrap approach of a backwards selection procedure. For four out of six time points we found IgG glycosylation to be the main or even only significant ‘predictor’. IgA glycosylation was only significant in the second and third trimester of pregnancy, with quite small effect sizes. Altogether, our data strongly suggests a more prominent association for IgG than for IgA with disease activity, which may imply a more prominent role for changes in IgG glycosylation in the pathogenesis of RA and in the improvement of RA during pregnancy.

In conclusion, we have demonstrated on a large dataset that there is a difference in IgA glycosylation between RA patients and healthy controls, and that the glycosylation of IgA
changes during pregnancy. However, most differences and changes were minor, so it is unlikely that these have any biological significance. In addition, only minor associations of IgA glycosylation with RA disease activity were observed. This all suggests only a limited role for IgA glycosylation in the pathogenesis of RA, which is in sharp contrast to what has been previously shown for IgG glycosylation. Nevertheless, other properties of IgA may still be important in the pathogenesis of RA, such as the level of IgA plasmablasts.³⁴
References

1. de Man YA, Bakker-Jonges LE, Goorbergh CMD-vd, et al. Women with rheumatoid arthritis negative for anti-cyclic citrullinated peptide and rheumatoid factor are more likely to improve during pregnancy, whereas in autoantibody-positive women autoantibody levels are not influenced by pregnancy. Ann Rheum Dis 2010;69(2):420-23.


CHAPTER 8

REDUCED INCREASE OF ACPA IgG GALACTOSYLATION DURING PREGNANCY IN COMPARISON TO TOTAL IgG: AN EXPLANATION WHY AUTOANTIBODY POSITIVE RA-PATIENTS IMPROVE LESS DURING PREGNANCY?

Albert Bondt, Lise Hafkenscheid, David Falck, T. Martijn Kuijper, Yoann Rombouts, Johanna M.W. Hazes, Manfred Wuhrer, Radboud J.E.M. Dolhain

Manuscript in preparation.
Abstract

Objectives: Rheumatoid arthritis (RA) disease activity (DAS28-CRP) improves less during pregnancy in autoantibody positive patients. Anti-citrullinated protein antibodies (ACPAs) are the most specific autoantibodies for RA. Total immunoglobulin (Ig) G glycosylation is associated with DAS28-CRP and the pregnancy-associated improvement thereof. ACPAs – mainly present as IgG – are different from total IgG with respect to their glycosylation. Therefore, we sought to determine whether changes in ACPA IgG glycosylation provide an additional insight in the mechanism behind the pregnancy-associated improvement of RA.

Methods: ACPA positive patient sera ($n=152$) were obtained within the framework of the PARA cohort, a prospective study designed to investigate pregnancy-associated improvement of RA. ACPA IgG was isolated using microscale affinity chromatography. Trypsin digested ACPA IgG was measured using nano-liquid chromatography mass spectrometry, and compared to total IgG.

Results: Pregnancy-associated changes in the levels of galactosylation were observed for all ACPA IgG subclasses. Pregnancy-associated glycosylation changes were more pronounced in total IgG compared to ACPA IgG, but for total IgG not different between ACPA+ and ACPA- patients. No association of the change in DAS28-CRP with the change in ACPA IgG or total IgG galactosylation was observed for ACPA positive patients, whereas a strong association of total IgG galactosylation was observed for ACPA negative patients.

Conclusions: For ACPA IgG and total IgG in ACPA+ patients no associations with DAS28-CRP were observed, in contrast to total IgG galactosylation in ACPA- patients, for which also differences between responders and non-responders were observed. This suggests a different role for antibodies and/or antibody glycosylation in autoantibody positive versus negative RA.
Introduction

In rheumatoid arthritis (RA) autoantibodies (AAbs) are considered important factors in the pathogenesis of the disease. A wide variety of AAbs has been discovered in RA over the years, but rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are found in the majority of RA-patients.\textsuperscript{1} For both ACPA and RF the IgM, IgG and IgA isotype have been recognized.\textsuperscript{2,3} An increasing body of evidence suggests that AAb positive RA is different from AAb negative RA. For example, it has been shown that AAb positive RA is more progressive and destructive, in particular when ACPA is present.\textsuperscript{3} In addition, while approximately 50\% of all RA-patients spontaneously improve with pregnancy, this percentage is lower for AAb positive disease, and often disease activity in these patients remains moderate to high.\textsuperscript{4,5}

Immunoglobulin (Ig) G is known to bear sugar structures at asparagine 297 of the constant fragment (fragment crystallisable; Fc) of the heavy chain, the so called \textit{N}-glycan. These \textit{N}-glycans on IgG are generally composed of a core structure of seven building blocks (four \textit{N}-acetylglucosamines (GlcNac) and three mannoses), which can be extended by galactoses, sialic acids, a fucose and a bisecting GlcNac (Figure 1). For ACPA IgGs it has furthermore been described that in addition glycans with only three GlcNacs in the core structure, called mono-antennary glycans, can be found.\textsuperscript{6}

In particular the absence of galactose-extensions is known for decades to be associated with higher RA disease activity, whereas the increase in the levels of galactose is associated with the spontaneous improvement of RA during pregnancy.\textsuperscript{7,8} The glycan moiety may modulate the IgG effector functions by influencing Fc-gamma-receptor binding, or by activating anti-inflammatory pathways via lectins such as Dectin-1.\textsuperscript{9,10} Because of these and other observations IgG-Fc \textit{N}-glycans without galactose are often called to have a ‘pro-inflammatory’ phenotype, and those with galactose ‘anti-inflammatory’, although this is mainly based on associations and not functional studies.

In RA AAbs, in particular ACPA and rheumatoid factor, can be present years before disease onset without clinical phenotype, but their mere presence is not enough.\textsuperscript{11} However, shortly before diagnosis of RA the ACPA IgG Fc \textit{N}-glycosylation is changed in appearance to the phenotype associated with increased inflammation: towards the so-called agalactosylated glycans, with low levels of galactosylation and sialylation.\textsuperscript{6,12,13}

\textbf{Figure 1. Schematic representation of an \textit{N}-glycan.} The stable core is indicated by the dashed line. The dotted blue line indicates the truncation called mono-antennary. The four possible extension classes of the glycan are furthermore shown: 1) sialylation, 2) galactosylation, 3) bisection (for ‘bisecting’ \textit{N}-acetylglucosamine), and 4) fucosylation.
Since ACPA IgG has been shown to possess a more ‘pro-inflammatory’ Fc-glycosylation profile compared to total IgG, and AAb positive RA-patients are less likely to improve during pregnancy, we hypothesized that this might be related to the fact that auto-antibodies, like ACPA, are more likely to retain their pro-inflammatory glycosylation profile during pregnancy compared to total IgG.\textsuperscript{4,5,14} Therefore we compared the changes in the glycosylation of ACPA IgG upon pregnancy and after delivery with those of total IgG. In addition associations with changes in disease activity were investigated. Finally, changes in total IgG glycosylation data were compared between ACPA+ and ACPA- patients.

Methods

Study population and clinical response

The current study is embedded in the PARA (Pregnancy-induced Amelioration of Rheumatoid Arthritis) study, a nationwide prospective cohort study on pregnancy and RA for which patients were included between 2002 and 2009, and sera were collected pre-conception if possible, at three time points during pregnancy and three time points after delivery.\textsuperscript{15} All patients fulfill the 1987 ACR criteria for RA. The study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the Erasmus University Medical Center, Rotterdam, The Netherlands. At all time points disease activity was assessed using the DAS28 score based upon C-reactive protein (CRP), and swollen and tender joint count.\textsuperscript{16} Responders and non-responders (from pre-conception to third trimester, or from first to third trimester) were categorized based upon the EULAR response criteria.\textsuperscript{17} The postpartum flare was defined between the third trimester and 12 weeks postpartum using the so-called “reversed EULAR response criteria”.\textsuperscript{15} Patient characteristics as well as clinical response outcomes are depicted in Table 1.

Total and ACPA IgG glycosylation data

ACPAs were captured and processed as described before, with minor modifications.\textsuperscript{18} Briefly, ACPAs were affinity purified from 25 μL human serum using NeutrAvidin beads coated with CCP2 peptide, in a 96-wells format. In addition to the patient samples several ACPA negative healthy controls were included. Eluates from the CCP2 beads were immediately neutralized using 0.2 M Tris buffer. The neutralized ACPAs were then subjected to a Protein G affinity capturing step to enrich for IgG. The Protein G eluates were dried by vacuum centrifugation, followed by reconstitution in 50 mM ammonium bicarbonate (Sigma Aldrich, Steinheim, Germany) with 15% acetonitrile. As a next step, the same volume of ultrapure water containing 600 ng of TPCK treated trypsin (Sigma Aldrich) was added before overnight digestion at 37 °C. The obtained tryptic digests were then measured by nano-LC-ESI-MS as described before.\textsuperscript{19} Total IgG glycosylation data obtained previously was used to compare with the ACPA IgG glycosylation.\textsuperscript{8}
Data analysis
DataAnalysis version 4.2 (Build 395; Bruker Daltonics) was used for the visualization of a variety of IgG glycoforms for each subclass to obtain retention times. The raw LC-MS data was then converted to mzXML using MSConvert. LaCyTools was used to align the LC runs, extract and calibrate the mass spectra of each IgG subclass, and integrate the spectra for a selected list of potential analytes. In addition, several quality measures were extracted. After analyte and spectrum curation, several glycosylation traits were calculated in Microsoft Excel as described in the Supplemental Material. The ACPA negative sera were used to assess the specificity of the entire procedure. An additional threshold for inclusion was set at two times the standard deviation of the subclass specific intensity obtained for these negative sera.

Statistical analysis
Statistical analysis was performed using Stata/SE 13.1 for Windows (StataCorp LP, TX, USA). First all ACPA IgG and total IgG time lapses of subclass specific glycosylation traits, as well as disease activity, were modelled using multilevel mixed-effects linear regression analysis, similar to what has been described before. In addition, for total IgG separate models were estimated for ACPA positive and ACPA negative patients. Within these models the presence of pregnancy-associated changes were studied between pre-conception (PC) and third trimester (TM3), between first trimester (TM1) and TM3, and between TM3 and the second postpartum time point (12 weeks postpartum; PP2). For levels of bisecting GlcNAc an additional test was performed from PC to TM1. (The selection of time points to compare was driven by the time lapses of the glycosylation traits and the disease activity score.) Delta (Δ) values were calculated for the changes in the glycosylation traits as the value at TM3 minus the value PC or at TM1 for the change during pregnancy, and as the value at PP2 minus the value at the TM3 for the change after delivery. It was tested whether the mean ΔACPA IgG glycosylation were different as compared to the total IgG glycosylation changes, and whether Δtotal IgG glycosylation were different between ACPA+ and ACPA-patients. Two-group mean-comparison tests were performed to identify possible differences between responders and non-responders (during pregnancy), or “flare” and “no flare” (after delivery). Corrections for multiple testing were applied as depicted in the separate results sections.

Results
Study population and clinical response
Sera from the 152 ACPA positive patients included in the PARA cohort (total n=255) with material available from at least three time points were used for this study. For the entire cohort (both ACPA positive as well as ACPA negative patients) material was available of 130 patients from pre-conception, 97 patients were followed from 1st trimester, sixteen patients from 2nd trimester, and twelve from 3rd trimester onwards. After sample processing analyses
could be performed on ACPA IgG glycopeptide data from 112 ACPA positive patients: 50 from pre-conception onwards, 48 from the 1st trimester, nine from the 2nd trimester, and five from the 3rd trimester. Patient characteristics are depicted in Table 1.

Table 1. Cohort characteristics

<table>
<thead>
<tr>
<th></th>
<th>ACPA+</th>
<th>ACPA+ with spectra</th>
<th>ACPA-</th>
<th>ACPA+ vs ACPA-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean [range]</td>
<td>33.1</td>
<td>33.1 [21.9-42.4]</td>
<td>32.3</td>
<td>ns</td>
</tr>
<tr>
<td>RFpositive, n (%)</td>
<td>133/146 (91)</td>
<td>95/105 (90)</td>
<td>28/94 (30)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Erosive disease, n (%)</td>
<td>101/148 (68)</td>
<td>74/107 (69)</td>
<td>49/99 (49)</td>
<td>0.0037</td>
</tr>
<tr>
<td>DAS28-CRP3, mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>3.6 (1.2)</td>
<td>3.8 (1.2)</td>
<td>3.6 (1.0)</td>
<td>ns</td>
</tr>
<tr>
<td>TM1</td>
<td>3.8 (1.2)</td>
<td>3.9 (1.2)</td>
<td>3.3 (1.0)</td>
<td>0.0002</td>
</tr>
<tr>
<td>TM3</td>
<td>3.5 (1.1)</td>
<td>3.5 (1.0)</td>
<td>3.0 (1.0)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Use of sulfasalazine at TM1, n (%)</td>
<td>37/135 (27)</td>
<td>31/96 (32)</td>
<td>26/89 (29)</td>
<td>ns</td>
</tr>
<tr>
<td>Use of prednisone at TM1, n (%)</td>
<td>51/135 (38)</td>
<td>37/96 (39)</td>
<td>29/89 (33)</td>
<td>ns</td>
</tr>
<tr>
<td>Use of hydroxychloroquine at TM1, n (%)</td>
<td>3/135 (2)</td>
<td>3/96 (3)</td>
<td>2/89 (2)</td>
<td>ns</td>
</tr>
<tr>
<td>Use of methotrexate in the past, n (%)</td>
<td>87/152 (57)</td>
<td>63/112 (56)</td>
<td>53/101 (52)</td>
<td>ns</td>
</tr>
<tr>
<td>Use of TNF blocking agents in the past, n (%)</td>
<td>23/152 (15)</td>
<td>18/112 (16)</td>
<td>10/101 (10)</td>
<td>ns</td>
</tr>
<tr>
<td>Response during pregnancy, n (%)</td>
<td>14/32 (44)</td>
<td>9/23 (39)</td>
<td>20/34 (59)</td>
<td>ns</td>
</tr>
<tr>
<td>Flare after delivery, n (%)</td>
<td>35/74 (47)</td>
<td>29/55 (53)</td>
<td>21/44 (48)</td>
<td>ns</td>
</tr>
</tbody>
</table>

1 Abbreviations used: ACPA = anti-citrullinated protein antibodies; ACPA+ = ACPA positive; ACPA- = ACPA negative; RF = rheumatoid factor; PC = pre-conception; TM = trimester; PP = postpartum; ns = not significant.
2 Outcome of the t-test comparing ACPA+ with spectra to all ACPA- patients. Similar results were obtained for comparing all ACPA+ to all ACPA-.
3 ACPA+ n=65; ACPA+ with spectra n=46; ACPA- n=52
4 ACPA+ n=129; ACPA+ with spectra n=91; ACPA- n=85
5 ACPA+ n=138; ACPA+ with spectra n=97; ACPA- n=96
6 This number is lower than the total number of patients since some patients were included later than TM1.
7 The EULAR response criteria require an initial DAS28>3.2 and can thus be applied only on a subset of patients.
ACPA glycosylation shows pregnancy-associated changes

An increase of ACPA IgG galactosylation was observed between the first and third trimester of pregnancy for all subclasses (IgG1 +3.40%, p<0.001; IgG2/3 +5.01%, p<0.001; IgG4 +3.99%, p<0.001), followed by a decrease after delivery (IgG1 -4.39%, p<0.001; IgG2/3 -5.89%, p<0.001; IgG4 -5.09%, p<0.001; Figure 2, Table 2, Supplemental Tables 1 and 2).

Of note, it appears that the postpartum decrease of ACPA IgG4 galactosylation starts later compared to ACPA IgG1 and -IgG2/3.

Furthermore, for ACPA IgG also sialylation showed an increase during and decrease after pregnancy (Table 2, Supplemental Tables 1 and 2). Levels of bisecting GlcNAc were registered for ACPA IgG1 and ACPA IgG2/3 showing a drop between pre-conception and the first trimester, followed by a gradual increase during pregnancy which continued postpartum. Levels at third trimester were not different from the pre-conception time point (Supplemental Tables 1 and 2).

Afucosylated structures showed a mild decrease after delivery for ACPA IgG1. No afucosylated species were detected for ACPA IgG2/3 and ACPA IgG4 (Supplemental Tables 1 and 2). In addition to the previously studied glycosylation traits we were able to detect glycopeptides with mono-antennary glycans (<1% of all detected ACPA IgG1 glycopeptides), of which the level decreased during pregnancy (from 0.8 to 0.7%, p<0.001) and increased after delivery (from 0.7 to 0.8%, p=0.001; Supplemental Tables 1 and 2).

Figure 2. Galactosylation time curves for IgG1, IgG2/3 and IgG4. The ACPA glycosylation (red line, circle markers) is different as compared to total IgG of ACPA+ individuals (yellow line, triangle markers). In addition total IgG glycosylation patterns for ACPA- patients (grey line, square markers) and healthy controls (blue line, cross markers) are shown. Error bars represent standard errors.
Table 2. Comparison of the change in glycosylation (%) between ACPA IgG and total IgG. P < 0.006 was considered statistically significant after multiple testing correction.

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<tr>
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<td>61</td>
<td>-0.35</td>
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</tbody>
</table>

1 Abbreviations used: PC = pre-conception; TM = trimester; PP = postpartum; ACPA = anti-citrullinated protein antibodies; SE = standard error; Gal = Galactosylation; SA = Sialylation; Bis = Bisection; Fuc = Fucosylation.
Pregnancy-associated changes in glycosylation of ACPA IgG are less pronounced in comparison to total IgG

The change in galactosylation of IgG1 was on average 2-fold smaller for ACPA IgG compared to total IgG for all studied time spans (p<0.001; Figure 3A, Table 2). The increase during pregnancy and decrease after delivery was significantly lower for ACPA IgG2/3 as compared to total IgG2/3 (p<0.001), between PC and TM3 as well as between TM3 and PP2, but not between TM1 and TM3 (p=0.266). The decrease of galactosylation of ACPA IgG4 after delivery was less compared to that of total IgG4 (p=0.004), whereas the increase during pregnancy did not reach statistically different levels (p>0.07).

A highly comparable set of observations was obtained for sialylation, with a less pronounced increase during pregnancy and postpartum decrease in sialylation of ACPA IgG1 and ACPA IgG2/3 in comparison to total IgG for those subclasses (Fig. 3B; Table 2). For IgG4 similar trends were observed.

The level of bisection decreased between PC and TM3 for total IgG1, while a slight increase was observed for ACPA IgG1 bisection. From TM1 to TM3 an increase was observed for both total and ACPA IgG1, which was more pronounced for ACPA. After delivery the increase continued, but in this time frame it was more pronounced for total IgG1. Intriguingly, for IgG2/3 the change between TM3 and PP2 was more pronounced in ACPA IgG. The other studied changes over time of IgG2/3 bisection did not show differences between total and ACPA IgG (Table 2).

Figure 3. Pregnancy-associated changes in IgG1 galactosylation (A) and sialylation (B) are more pronounced in total IgG compared to ACPA in the same patients. Changes from pre-conception (PC) to the third trimester (TM3), from first trimester (TM1) to TM3, and from TM3 to the second postpartum time point (PP2; 12 weeks postpartum) are shown for total IgG in blue and ACPA-IgG in orange. (*** p<0.001)
Highly comparable glycosylation changes for total IgG from ACPA+ and ACPA- patients

The increase in total IgG galactosylation during pregnancy was not different between ACPA+ and ACPA- patients, and neither was the decrease postpartum (Supplementary Table 3). For sialylation there was similarly no difference in the increase during pregnancy. However, postpartum IgG1 sialylation showed a more pronounced decrease in ACPA- as compared to ACPA+ patients (-3.0% vs -2.5%; p=0.0048; Supplementary Table 3). Interestingly, in total IgG1 of both ACPA+ and ACPA- patients a similar decrease in bisection upon conception was observed, but no increase to the third trimester, while pre-conception levels were again reached after delivery (Supplementary Tables 1, 2 and 3). Of note, the time lapse of total IgG2/3 bisection of ACPA+ patients, but not ACPA- patients, was similar to the time lapse of ACPA IgG2/3 bisection.

Fucosylation of total IgG1 mildly decreased during pregnancy and increased after delivery for both ACPA+ and ACPA- patients (Supplementary Tables 1 and 2).

The association of galactosylation with disease activity is not observed for ACPA IgG, and neither in total IgG of ACPA+ patients

We previously found that the change in total IgG galactosylation is associated with the change in disease activity. Regression analysis was now performed to study this phenomenon for ACPA IgG. In addition, for total IgG a subgroup analysis for ACPA+ and ACPA- patients was performed.

No ACPA IgG galactosylation changes were found to associate with changes in disease activity (Table 3). Only for ACPA IgG1 a nearly significant negative association between the changes from TM1 to TM3 was observed (p=0.0058 (p<0.0056 considered significant after Bonferroni correction), $R^2=0.13$, $\beta=-0.36$).

For total IgG galactosylation in the ACPA+ population also no significant association was observed. On the other hand, the total IgG galactosylation of ACPA- patients showed strong negative associations for both IgG1 and IgG2/3, during pregnancy (TM1-TM3, p=0.001) as well as after delivery (TM3-PP2, p<0.001; Table 3).

Furthermore, these analyses were performed for changes in sialylation. Again, no associations of ACPA IgG with changes in disease activity were observed (Table 3). Also, the changes in total IgG sialylation for ACPA+ and ACPA- patients did not show significant associations with disease activity (Table 3).
Table 3. Association of the change in galactosylation and sialylation with the change in disease activity during different time spans for ACPA IgG and total IgG in the ACPA+ patients, and for total IgG in the ACPA- patients. P<0.006 is considered statistically significant after multiple testing correction.

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1 Abbreviations used: PC = pre-conception; TM = trimester; PP = postpartum; ACPA = anti-citrullinated protein antibodies; ACPA+ = ACPA positive; ACPA- = ACPA negative.
ACPA IgG and total IgG glycosylation of ACPA+ patients do not differ between clinical response groups, whereas total IgG glycosylation of ACPA- patients does

Previously we have shown that changes in glycosylation are more pronounced in patients that respond during pregnancy and those that flare after delivery. To determine whether this is related to ACPA status a subgroup analysis was performed.

Patients were classified as ‘responder’ and ‘non-responder’ during pregnancy, or ‘flare’ and ‘no flare’ after delivery. For ACPA IgG1 galactosylation was found to increase slightly more in responders compared to non-responders (p=0.044; Table 4). Total IgG galactosylation in ACPA positive patients however did not show different increases between responders and non-responders during pregnancy, nor a difference in decrease between patients with and without a flare after delivery. Interestingly, a nearly 2-fold larger increase in total IgG1 galactosylation was observed in the ACPA- responders (+8.9%) during pregnancy as compared to the non-responders (+4.8%; p=0.005; Table 4). After delivery the decrease in total IgG1 galactosylation was 1.5-fold more pronounced in patients with a flare of disease activity (-13.2% vs. -9.3%; p=0.001). Similar findings were observed for total IgG2/3 (Table 4).

Although less pronounced, for sialylation almost the same was observed: ACPA IgG or total IgG sialylation in ACPA+ patients was not different between responders and non-responders or between patients with or without flare. In contrast, total IgG sialylation in ACPA- patients did show a modest difference (IgG1: p=0.03; IgG2/3: p=0.01) between patients without a flare (IgG1: -2.8±0.2%; IgG2/3: -3.1±0.2%) compared to patient with a flare of disease activity (IgG1: -3.4±0.2%; IgG2/3: -4.0±0.3%; Table 4). However, these findings did not reach significance after correction for multiple testing.
Table 4. Galactosylation and Sialylation for different clinical response groups. A comparison between responders and non-responders (from PC-TM3 or TM1 to TM3) or between patients with and without a flare of disease activity (between TM3 and PP2) was made. Depicted are the number of patients per group, the group mean and standard error, and the significance of the two means being equal. P-values highlighted in bold font are still significant after Bonferroni correction for 9 tests (p<0.006).

<table>
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<tr>
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| Sialylation         |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| ACPA IgG            |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| IgG1                | 11      | 0.85    | 0.54    | 20      | 0.44    | 0.19   | 50     | -1.04 | 0.15 | 19    | -1.28 | 0.24 | 0.402 |
| IgG2/3              | 5       | 0.89    | 0.95    | 9       | 1.47    | 0.98   | 22     | -1.63 | 0.20 | 8     | -1.59 | 0.53 | 0.935 |
| IgG4                | 2       | 2.56    | 1.20    | 14      | 1.17    | 0.42   | 23     | -1.95 | 0.38 | 12    | -1.80 | 0.49 | 0.817 |
|                     |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| Total IgG (ACPA+)   |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| IgG1                | 13      | 2.43    | 0.34    | 26      | 1.16    | 0.22   | 65     | -2.42 | 0.12 | 29    | -2.63 | 0.24 | 0.392 |
| IgG2/3              | 13      | 2.94    | 0.34    | 26      | 1.60    | 0.19   | 65     | -2.88 | 0.13 | 29    | -3.17 | 0.24 | 0.258 |
| IgG4                | 7       | 3.62    | 0.67    | 16      | 1.61    | 0.37   | 25     | -2.60 | 0.31 | 9     | -2.68 | 0.63 | 0.902 |
|                     |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| Total IgG (ACPA-)   |         |         |         |         |         |       |       |       |       |       |       |       |       |       |
| IgG1                | 14      | 3.19    | 0.31    | 19      | 3.21    | 0.32   | 964    | -2.76 | 0.16 | 27    | -3.38 | 0.23 | 0.031 |
| IgG2/3              | 14      | 3.59    | 0.28    | 23      | 1.98    | 0.16   | 62     | -3.15 | 0.17 | 27    | -3.96 | 0.26 | 0.011 |
| IgG4                | 6       | 4.26    | 1.47    | 6       | 5.14    | 0.92   | 62     | -3.06 | 0.45 | 8     | -4.05 | 0.76 | 0.261 |
Discussion

In the current manuscript we sought to determine whether the Fc glycosylation of autoantibodies could provide an explanation why RA-patients positive for these antibodies are less likely to improve during pregnancy. ACPA IgG was used as a model for this phenomenon. Indeed, we did observe that ACPA IgGs keep a glycan phenotype that is associated with higher inflammation, with lower galactosylation as a hallmark. In contrast, for total IgG of both ACPA+ and ACPA- patients a more than 2-fold stronger increase in galactosylation was observed with pregnancy. Notably, in ACPA- patients – but not ACPA+ patients – there was a strong association in the change in galactosylation of total IgG with the change of the disease activity.

The pregnancy-associated changes between ACPA IgG and total IgG are profoundly different, with less than 5% increase of galactosylation of ACPA IgG during pregnancy, whereas total IgG galactosylation in both ACPA+ and ACPA- patients increases with more than 10%. Thus, we demonstrated that a RA specific subset of (auto)antibodies only show a small increase in galactosylation during pregnancy, and hence retain an glycan phenotype that is associated with higher inflammation. This might be an explanation why autoantibody positive RA-patients do not show a similar pregnancy-associated improvement of disease activity as do autoantibody negative RA-patients. The reason why ACPA during pregnancy retain this more pro-inflammatory glycan phenotype is not entirely clear. It has been shown that IgGs produced in a pro-inflammatory environment, like ACPA in the inflamed synovium of RA-patients, possess a lower degree of galactosylation. Since the observed difference in galactosylation between ACPA and total IgG was mainly observed during pregnancy and not before or after delivery, this cannot account for the findings in this study.

As an increase in oestrogen levels is thought to be responsible for the increase in IgG galactosylation upon pregnancy, one could speculate that ACPA-producing plasma cells are less sensitive for this effect of oestrogens on glycosylation. If this is the case, this decreased sensitivity is unlikely to reside at the level of oestrogens receptor expression, because normal levels of hormone receptors are present and functional in RA synovium. Intriguingly, the inflammatory environment, with high levels of for example IL-6, is associated with increased levels of oestrogen receptors. It has been suggested that high levels of oestrogen possess pro-inflammatory effects, whereas low levels would be anti-inflammatory. The increased expression of oestrogen receptors, together with the increased level of oestrogen during pregnancy, may therefore result in an over-stimulated, hence pro-inflammatory, synovium. This may finally prevail any anti-inflammatory signalling induced by other hormones and causing (or being caused by) lower levels of (ACPAs-)IgG galactosylation. Therefore, effects of the increase of pregnancy hormones may result in differential glycosylation modifications. Alternatively, ACPA-producing B cells appear to be generally more matured with high rates of class-switched memory B cells and plasmablasts. In addition, these cells may be generated more often and persist longer in the synovium. These more matured or ‘older’ antibody
producing cells may be less sensitive to (hormonal) stimulation which would modify the IgG glycosylation. Such desensitisation has for example been reported for matured T cells.28

Another finding of this study is that the change in total IgG galactosylation during pregnancy and after delivery was similar between ACPA+ and ACPA- patients, while the association with disease activity was markedly different. We had expected that, due to the potentially pathogenic role of autoantibodies in ACPA+ patients, this association would have been more pronounced in these patients. However, we observed the opposite. In the ACPA+ patients neither the change in total nor ACPA IgG galactosylation associate with improvement of disease activity during pregnancy or the flare after delivery. In the ACPA- patients on the other hand, showing the same mean total IgG galactosylation increase as do ACPA+ patients, an association with the change in DAS28 was observed. These differences in association cannot be explained by different group sizes (Table 3). With regards to the postpartum flare an explanation may be that ACPA+ patients, generally experiencing a more severe disease activity, are back on medication earlier, which could have an impact on the association between the naturally occurring flare of disease activity and the decrease in galactosylation after delivery. This cannot explain the stronger association in the ACPA- patients of the change in galactosylation with the change in disease activity during pregnancy, since only in very few patients of this cohort medication was changed in that period. Alternatively, the association between ACPA-galactosylation and disease activity may not be strictly linear, but a certain “galactosylation threshold” may be involved: when the galactosylation is below this threshold the autoantibodies retain their destructive functions. For that reason, during entire pregnancy the glycan profile of ACPA would remain more pro-inflammatory, which may not only influence the association between the galactosylation of ACPA and disease activity, but also that of total IgG in ACPA+ patients.

Finally, the observation that ACPA glycosylation is not associated with a change in DAS28 could be interpreted as an argument for ACPA being a mere epiphenomenon, and not directly involved in RA pathogenesis. However, also in granulomatosis with polyangiitis (GPA; formerly known as Wegener’s) we have observed that autoantibody glycosylation is not associated with disease activity, whereas total IgG glycosylation does (Kemna et al., unpublished results). For GPA it is more established that the autoantibodies that are present, namely anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase-3 (PR3) or myeloperoxidase (MPO), are pathogenic and not an epiphenomenon, and that antibody glycosylation plays an important role.29-31 Intriguingly, in our present study, as well as in the mentioned GPA study, galactosylation appears to be more important than sialylation, which is in line with our previous findings, but in contrast to many mouse studies.8,32,33

In conclusion, we have shown that during pregnancy ACPA retains a more pro-inflammatory glycosylation profile compared to total IgG, which could be an explanation why auto-antibody positive RA-patients are less likely to improve during pregnancy. Furthermore, in
ACPA+ patients we found no association of changes in ACPA IgG or total IgG galactosylation with disease activity, whereas this was the case for patients with ACPA negative disease.

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Rheumatoid arthritis (RA) is a widespread autoimmune disease affecting approximately 1% of the Western population. Intriguingly, the disease is known to often improve during pregnancy, and a flare of disease activity may be observed after delivery. The mechanism by which the improvement and flare occur are currently unknown, but one of the potential answers lies with antibody glycosylation. The glycosylation of the constant domain of immunoglobulin (Ig) G is known to associate with disease activity in RA. Furthermore, several changes have been observed in association with pregnancy. The maternal immune system requires major adaptations to cope with the presence of a partially foreign fetus during pregnancy. It has been hypothesized that these adaptations cause the improvement of RA during pregnancy. Antibodies, as key players in autoimmunity, were suggested to be involved.

The research described in this thesis was performed to increase the understanding of mechanisms underlying the pregnancy-associated changes in disease activity, and to determine the potential roles of antibodies and their glycosylation in this process. In part, this was achieved by developing techniques that allow for more throughput and detailed compositional characterization of antibody glycosylation (Part II), opening the way to study large sample sets and be able to observe general effects usually blurred by large biological diversity. Then these methods were applied on the PARA cohort which has been designed for studying the interaction of pregnancy and rheumatoid arthritis (Part III). In this way, associations with clinical variables were examined, which could be a lead for generating hypotheses concerning mechanisms involved in the improvement of RA during pregnancy. Future research needs to be performed to falsify the thus generated hypotheses.

**Immunoglobulin G Fc**

In Chapter 5 we showed that galactosylation of IgG-Fc is associated with disease activity and the pregnancy-associated change thereof, independent of the sialylation. IgG antibody glycosylation has been shown to associate with disease activity quite some time ago; in particular, high levels of agalactosyl (G0) glycans, thus low levels of galactosylation, were associated with high disease activity, currently often referred to as ‘pro-inflammatory’. Furthermore, several studies have shown changes in the IgG glycosylation, in particular its galactosylation, during pregnancy and an association with improvement. In addition, both galactosylation and sialylation have been suggested to possess ‘anti-inflammatory’ properties, generally based on mouse models and cell assays. Our data suggest that in the human ‘in vivo’ system galactosylation may be more important than sialylation.

The first indications of involvement of G0 glycans were obtained using exoglycosidase ‘sequencing’. By sequential treatment of the (chemically) released glycans with exoglycosidases a rough estimation of the percentage of specific glycoforms could be established. In addition, a monoclonal antibody targeted against terminal GlcNAc residues has been used to detect the G0 glycoforms. Alternatively, the ratio between galactosylated (as detected by BS-II lectin) and agalactosylated (as detected by RCA-I)
glycans was calculated. These lectin-based studies already showed the great potential of IgG-Fc glycosylation analysis, specifically those searching for agalactosyl IgG. When patients were followed over time the level of agalactosyl IgG was found to be associated with more progressive disease, thus being a prognostic marker. Although sophisticated in their days, these techniques have some drawbacks. Lectins generally cannot distinguish between one or two galactoses. Therefore, the association of increased RA disease activity with G0 glycans could be detected, but the opposite association of improvement with the percentage of galactosylation could not. In addition, for many approaches the sialic acid content remains unclear, since most techniques did not allow for the detection of these negatively charged and labile sugars, which were therefore removed. Furthermore, all N-glycans from IgG molecules were removed, not taking into account the different identity of the N-glycans on the Fab domain of IgG (demonstrated in Chapter 3 and 6). The liquid chromatography with online mass spectrometric detection which we used could obtain data that was Fc-specific by detecting glycopeptides instead of released glycans. In addition, subclass-specific information was obtained, and the technique allowed for a sensitive detection of also the sialylated species. This combination led to the main finding that galactosylation of the IgG-Fc N-glycans is associated with disease activity independent of sialylation. The addition of sialic acid even appeared to dampen the effect of the galactoses.

This indication of a limited involvement of sialic acids is in contrast to a widely accepted hypothesis in the field of IgG glycosylation immunomodulatory properties: these days, many scientists believe that sialic acid is the driver of the anti-inflammatory activity of intravenous Ig (IVIg) observed in various animal and human studies. This hypothesis is based on data obtained by a lectin based method using SNA (Sambucus nigra agglutinin), which is described to bind specifically α2,6-linked sialic acids. IgGs from preparations that bound to SNA were suggested to possess a 10-fold increased anti-inflammatory effect as compared to non-SNA binding fraction, because of their sialylated Fc glycan. The anti-inflammatory effect is generally explained by binding to another receptor than the Fc-gamma-receptors, namely DC-SIGN (and its murine variant SIGN-R1) via sialic acids. The hypothesis is that in humans the binding of sialic acids to DC-SIGN induces a signaling pathway ultimately resulting in an upregulation of the inhibitory Fc-gamma-receptor (FcγR) IIb. Although the ‘sialic acid/DC-SIGN’-hypothesis is still favored by many scientists, others have shown that only a minor fraction of SNA-binding IgGs carries sialic acid on the Fc portion. In contrast, a strong enrichment of glycans on the Fab portion was obtained. Moreover, binding of IgG to DC-SIGN was shown not to involve any sialic acids. Furthermore, a recent review provides strong indications that caution needs to be taken when extrapolating the murine data to the human immune system. Alternative explanations could be that the sialic acid inhibits pro-inflammatory signals of glycans with terminal galactoses. Galactosylation results in increased binding to the activating FcγRIIIa, and increased antibody dependent cellular cytotoxicity (ADCC).
Furthermore, in addition to ADCC there is also complement-dependent cytotoxicity (CDC). For CDC it also has been shown that the removal of galactoses reduces the cytotoxicity, thus that the presence of galactoses would be pro-inflammatory. However, others have reported that galactoses may exert an anti-inflammatory effect by inducing a heterodimer of the inhibiting FcγR-Ilb and Dectin-1, generating an inhibitory signal.

Many of the so-called ‘pro-’ and ‘anti-inflammatory’ experimental findings are based on cellular assays. As an alternative to the cell-based assays, the effect of differential glycosylation has been interpreted from in vivo experiments in mice or under naturally occurring physiological changes in humans, with phenotypic changes as read-out. For the human system an example is the study of RA disease activity during pregnancy, where RA is the model for inflammation, and disease activity is the read-out. In our data obtained for the PARA-cohort increased galactosylation, but not sialylation, was associated with lower inflammation. This human ‘in vivo’ finding still requires confirmation in other longitudinal RA cohorts, and other (autoimmune) inflammatory diseases such as Guillain-Barré syndrome or Crohn’s disease. Alternatively, the findings could be challenged in humanized mice (with a human immune system), although one needs to take into account that there is evidence that more cell types than those of the immune system may be involved in IgG sialylation.

Solving the galactosylation or sialylation discrepancy is a relevant research exercise needed to prove the relevance of mouse models for the human immune system, and may furthermore provide a rational for developing more effective therapeutic monoclonal antibodies. However, the more challenging and relevant question remains to determine whether there is a functional role, and not just an association, for IgG-Fc glycosylation in inflammation, as discussed later in the ‘Future research’ section.

**Immunoglobulin G Fab**

In Chapter 3 and 6 we described the development and application of a specific Fab N-glycosylation analysis technique. Until then, no method for higher-throughput analysis of IgG-Fab glycosylation was available. Notably, the method developed by us allows for a thorough characterization of the glycan compositions. Most publications with regards to Fab glycosylation used the term “asymmetrical antibodies”. This terminology is based on another example of historical misinterpretation of lectin binding assays, involving the lectin Concanavalin A (ConA). The literature basis for the antibodies being asymmetrical arises from a study in which all F(ab’)2 portions bound to ConA, but only half of the Fab portions. This would suggest that on every IgG with glycosylation in the variable domain, this glycosylation is present on only one of the two Fab arms, thereby being asymmetrical. However, in contrast to the common assumption that ConA binds only high-mannose type glycans, the lectin has been shown to bind complex type diantennary glycans as well as high-mannose structures, and potentially also hybrid types. In addition, it has been indicated that specifically complex-type glycans without bisecting GlcNAc are recognized. Thus, the alternative explanation for the binding of only half of the Fab portions would be that
50% of the glycans present on the Fab have no bisecting GlcNAc. This explanation matches with our observations. Whether or not the Fab glycosylation is asymmetrical remains to be elucidated.

The Fab glycans are often suggested to be only or mainly of the high-mannose type. Again, this is based on ConA binding, which may in part be true, but not completely. In Chapter 3 we also observed a minor fraction of high-mannose type glycans, but not in Chapter 6. Taking all of our Fab glycosylation observations up to this date into account, we have come to believe that the observation of high-mannose type glycans on the Fab domain of IgG is not real, but more likely an artefact from contaminants from either the serum or the buffers and enzymes used.

Intriguingly, we observed clear associations of IgG-Fc glycosylation with RA disease activity while this was not the case for IgG-Fab, despite pregnancy-associated changes in the Fab glycosylation. Of note, for IgG-Fc we have shown that the increase in galactosylation is associated with the improvement of disease activity. On the Fab portion galactosylation is already nearly complete, leaving little room for an increase, and thereby no association can be found. Alternatively, since only approximately 17% of the Fab portions carries a glycan it is possible that the role of Fab glycans lies more in the presence/absence than in the modification of the glycan structure. For example, it has been shown that a high percentage of autoantigen recognizing ACPA-IgG carry Fab glycans. However, in contrast to the glycans on the Fc portion for which differences between ACPA IgG and total IgG are observed, the Fab glycans on ACPA IgG show compositions highly similar to total IgG. This suggests that on the Fab-part of the IgG molecule the increased presence of glycans, rather than the specific composition of these glycans, could be pathogenic. However, during pregnancy the presence of Fab glycans is thought to increase. Again, the increase during pregnancy reported by others is mainly based on ConA binding experiments. This could alternatively be explained by the observation of lower percentages of glycans with a bisecting GlcNAc during pregnancy, resulting in increased ConA binding. Using our novel mass spectrometric approach we could confirm that during pregnancy indeed the abundance of Fab N-glycans increased, albeit with a small percentage. In addition, the level of bisecting GlcNAc decreased during pregnancy, which may also account for a part of the increase in ConA binding reported earlier. However, the increase in presence of Fab glycans is not associated with the change in disease activity.
Interestingly, also in another inflammatory setting evidence is provided that the presence of Fab glycans may be relevant for the outcome of an immunological process. In the recent study by Lu et al., digalactosylated (G2) IgG glycans were the main discriminant between latent and active tuberculosis patients, but ‘disialylation’ and the G2S2 glycoform were also important according to the analysis. From the method described in Chapter 3 (and 6) of this thesis we know that it is extremely likely that these highly sialylated features are mainly Fab-derived. The samples from the patient group with the highest G2 and sialylation also exerted the highest ADCC. It is not clear how the Fab glycans exactly can be involved in ADCC. Molecular modeling data suggests that binding of IgG-Fc to its FcγRs occurs close to the IgG hinge region, in such a way that one can assume that the Fab portion may interfere, especially when large sialylated glycans are present (Figure 2). Of note, these Fc-FcγR interactions are modeled with Fc-only structures which may be different from intact IgG binding.

Figure 1. Schematic representation of an IgG molecule. The N-glycan on the Fc portion is shown at N297, whereas the N-glycan on the Fab portion can be anywhere in the variable domain of both heavy and/or light chains as indicated by NXXX.

This figure was originally published in Bondt et al., Immunoglobulin G (IgG) Fab Glycosylation Analysis Using a New Mass Spectrometric High-throughput Profiling Method Reveals Pregnancy-associated Changes. Mol Cell Proteomics. 2014; 13:3029-3039. © the American Society for Biochemistry and Molecular Biology, and slightly adapted for the current purpose.
Immunoglobulin A
Since autoantibodies in RA are present in multiple isotypes, at least IgA, IgG and IgM, we have developed a method to study IgA glycosylation (Chapter 4) and applied this to the PARA cohort to study the association with RA and pregnancy (Chapter 7). In our MALDI approach we could only detect IgA1 glycopeptides (one of which is identical between IgA1 and 2).

Despite some reports describing associations of IgA O-glycosylation with disease activity, we did not observe this. Furthermore, we were able to detect IgA1 N-glycopeptides, none of which associated with disease activity. Nevertheless, remarkable similarities in the pregnancy-associated changes were observed between IgA and IgG, albeit at different levels. For example, sialylation of the glycans on Asn144 showed a pregnancy-associated increase and a decrease after delivery. This suggests that the same mechanisms affect both IgA and IgG producing plasma cells. The larger technical variation in the measurements of IgA glycopeptides compared to those of IgG may in part explain why we did not find disease activity associations for IgA.

ACPA-IgG Fc
In Chapter 8 the same LC-MS method as described in Chapter 5 was used (with some upgrades), this time applied on ACPA-IgGs. We found that the increase in galactosylation of ACPA-IgG is much lower as compared to total IgG, which may explain why autoantibody positive patients improve less during pregnancy. Furthermore, an association of the change

Figure 2. IgG-Fc to FcγR (CD64; FcγRIIlα) binding. IgG1 binds these receptors through the crystallizable fragment (Fc) and recognizes antigens through the antigen-binding fragment (Fab). Published in: Ganesh P. Subedi; Adam W. Barb; mAbs 2016, 8, 1512-1524
in total IgG galactosylation with the change in disease activity was only observed for the ACPA negative patients, and not for the ACPA positive.

Similar to what we observed for (total) IgG-Fab and IgA glycosylation, no associations of the change in disease activity with the change in glycosylation was found, although ACPA-IgG1 galactosylation reached borderline significance. The limited number of samples (<70) may play a role in this lack of significance, since the corrected coefficient of this association is similar for ACPA-IgG1 and total IgG1 galactosylation. Subgroup analysis for ACPA positivity was performed on the total IgG-Fc glycosylation data obtained in the context of Chapter 5, showing striking differences: with similar group sizes for ACPA+ and ACPA- patients (n=82-94) the latter showed strong associations between the change in disease activity and the change in galactosylation, and the former did not. Also, the association with the change in disease activity in the ACPA negative population was strong and significant for galactosylation, whereas this was not the case for sialylation. While one would expect a stronger association of antibody glycosylation with disease activity in autoantibody positive disease, we did not observe this. The mechanism behind this counterintuitive finding remains to be elucidated.

**RA, glycosylation, and genetic risk factors**

Ever since the discovery of aberrant glycosylation in RA it has been suggested that RA is a disorder of glycosylation. However, our results of the Fab glycosylation analysis (Chapter 6) indicate that the glycosylation machinery of IgG producing plasma cells of RA patients may hardly be affected in disease, and that the pronounced differences observed for the IgG-Fc portion maybe protein- and site-specific. Additional proof is given by a recent genetics study, in which no association was found between RA and genes involved in IgG N-glycosylation. For other autoimmune diseases this meta-analysis has not been performed, so the potential association of glycosylation genes with autoimmunity still stands.

Similarly, it has been shown that there is no association between the relative level of the agalactosylated glycan and any genetic signature. However, several other genetic risk factors for RA have been found which may indirectly impact the glycosylation, for example in interleukin (IL-6) and its receptor, IL-6R. IL-6 is a pro-inflammatory factor, with increased levels in RA which are associated with more bone erosion. In this more severe disease one would expect lower levels of IgG galactosylation. Indeed, in the PARA cohort we observed a clear negative association of IL-6 with total IgG (1 and 2/3) galactosylation (3rd trimester data; unpublished results). IgG released glycan galactosylation similarly negatively associated with IL-6 titers. Also a strong negative association was found with ACPA-IgG2/3 galactosylation, and to a lesser extent with ACPA-IgG1 galactosylation. However, when separating ACPA+ and ACPA- patients, only for the latter an association between total IgG1 galactosylation and IL-6 titer was observed.

Several single nucleotide polymorphisms (SNPs) have been identified in and around the IL-6 and IL-6R genes. Some SNPs appear to be protective, whereas others are associated with more severe disease. There are protective IL-6R SNPs that decrease membrane-
bound IL-6R and thereby results in reduced IL-6 responsiveness of T-cells. The decrease in membrane-bound IL-6R leads to increased soluble IL-6R. Interestingly, in another study actually a positive association between sIL-6R and disease activity was observed (in ACPA+ patients). This soluble IL-6R may scavenge IL-6, thus reducing its pro-inflammatory effect and the negative association with IgG galactosylation.

One of the available biologic drugs to treat RA is tocilizumab, an IL-6R blocking antibody. Treatment has been found to reverse the bone erosion mentioned before. The association with IL-6 described above suggests that a reduction in IL6-R activation could induce an increase in galactosylation. In experimental settings the in vitro blocking of IL-6R decreases IL-21 production by activated CD4+ T-cells. Interestingly, in vitro stimulation of B cells with IL-21 resulted in increased galactosylation. Since a blocking of IL-6R decreases IL-21, and thus IL-21 cannot exert its positive effect on galactosylation, it seems that this treatment would reduce galactosylation, which is in contrast to what is suggested by the IL-6 association data.

In pregnancy a reduction in IL-6 levels is observed, which should result in less IL-6R activation. Although it is theoretically the same mechanism as described above for anti-IL-6R treatment, in pregnancy increased galactosylation is observed. The difference in response may of course be explained by the simple in vitro system compared to the complex in vivo pregnancy ‘system’. The complexity of the interplay between several factors is furthermore demonstrated by differences in response between IL-6 genotype variants on rituximab (anti-CD20) or etanercept (TNFα blocker), which are not directly linked to IL-6 itself or its receptor.

**Concluding remarks**

The data presented in this thesis strongly indicate that the improvement of RA disease activity during pregnancy is associated with total IgG-Fc glycosylation, and not with IgG-Fab glycosylation, IgA glycosylation or antigen-specific IgG-Fc glycosylation. Although the galactosylation of ACPA was not associated with improvement of RA during pregnancy, interestingly, the increase in galactosylation of ACPA was less than that of total IgG, which might at least partially explain why ACPA positive patients improve less during pregnancy than ACPA negative patients. The translation from the association to the real mechanism, however, is not yet elucidated. It is clear that IgG-Fc glycosylation impacts many antibody effector functions such as FcγR binding, and that it impacts cell based assay outcomes. Furthermore, there are obvious differences in IgG-Fc glycosylation between healthy controls and diseased individuals, as well as between several pathologies. The relation between these observations, however, is not so straightforward. Most of the observations are associative, and no cause or effect has been proven, and neither has been a clear pro- or anti-inflammatory role for any glycan composition. These terms should therefore be used with more care in IgG glycosylation studies.
Genetic studies so far have not shown that there lies a high risk of developing RA in genes directly associated with N-glycosylation. Three main hypotheses therefore remain to be studied:

**a)** there is some environmental factor, such as food, drugs or chemical exposure, or bacterial or viral infection, causing aberrant (IgG-Fc) glycosylation, which in turn may result in a variety of pathological effects,

**b)** there are genetic risk factors involved in glycosylation that we do not know of causing aberrant glycosylation and pathological effects, or

**c)** glycosylation becomes modulated by several inflammatory factors and is merely an innocent bystander. (This innocent bystander however may still prove to be a highly relevant prognostic factor.)

**Future research**

The underlying question in all hypotheses posed above is whether IgG-Fc glycosylation influences inflammation (**a** and **b**) or is influenced by inflammation (**c**). Independent of the outcome of these hypotheses IgG-Fc glycosylation, in particular the galactosylation, may prove to be a relevant predictor or prognostic marker in the clinic.

Studies on the performance of IgG galactosylation as a predictor of disease, or as a prognostic marker, could be established relatively easy and would therefore be the preferred first line of research. First of all, the potential was already established previously in a small cohort. In addition, early screening for changes in IgG-Fc glycosylation upon treatment, such as anti-IL6R, anti-CD20 or TNFα inhibitors, may indicate whether a patient will respond or not. If these changes are detectable, this may potentiate early steering of treatment. Longitudinal studies covering this topic should be designed, or the total IgG glycosylation could be examined in existing studies on the effect of treatment. Current technical advances seem to allow for studying IgG-Fc galactosylation from the total serum/plasma N-glycome, requiring less advanced technology as compared to specific IgG-Fc glycopeptides. Once its potential is demonstrated, the total plasma/serum N-glycome analysis could be developed to an easy and fast screening assay for the clinic. Furthermore, the longitudinal application of the technique to study for example the treatment response would meet the increasing demand for personalized medicine. Alternatively, released N-glycans from purified total IgG could be used. In addition to the IgG-Fc galactosylation status, the total IgG N-glycans provides an estimation of the level of Fab glycosylation, which also appears to be an RA risk factor or prognostic marker. More evidence for the latter statement still needs to be provided, but the Fab glycosylation method developed in the context of this thesis enables the required studies.

The main three hypotheses posed above are focused on whether the IgG glycosylation modification, either by external (hypothesis **a**) or internal factors (hypothesis **b**), causes
Inflammation, or that the glycosylation is modified by inflammatory circumstances (hypothesis c). Since decreased galactosylation has been found in many autoimmune and chronic inflammatory diseases it is a highly relevant topic. For RA smoking has been reported as an important risk factor, and also obesity. Whether the intake of smoke or a high fat diet (external factors) are able to trigger arthritis could be studied in vivo on for example mouse models. Furthermore, the high prevalence of autoimmunity in females suggests the involvement of hormonal factors. Thus, on a genetic level common denominators in the hormone system should be identified. Sequentially, in cell-based assays of immune cells, such as ADCC and CDC assays, the regulatory role of hormones may be studied, carefully making a distinction between male and female donor derived material. Similar conditions could be studied in mice with a fully humanized immune system to obtain insight in a more complete picture, including downstream effects. In addition, it could be studies whether the triggering of specific pathways have an effect on the glycosylation of antibodies derived from in vitro production systems. In particular pathways which contain potential risk factors for RA (such as IL-6R) should be examined. Factors with a strong impact on the antibody glycosylation could sequentially be applied to previously mentioned cell-based assays to study the downstream IgG-Fc effector function, and the effects on in vivo models. When no evidence for both hypothesis a and b can be found it would become more likely that hypothesis c is correct.

Increased understanding of downstream effects of aberrant glycosylation may provide additional insights and tools to further study the cause of the deviating glycan phenotypes. Association studies may aid in this process. For example, analyzing antigen-specific antibodies, comparing an established pathogenic species with a ‘general’ specific antibody, e.g. anti-tetanus, could indicate the importance of glycosylation state of specific antibodies. However, despite their limitations human cell-based immune assays on for example ADCC and CDC may better explain the real effect of observed alterations in glycosylation. Multiple variants of these assays should always be performed, using several single types of immune cells, but also on mixes of these types and on complete PBMCs, to more closely resemble the human in vivo interaction between cells. These studies together may in the end provide conclusive data on downstream effects actually caused by aberrant glycosylation observed in a pathology such as RA. In addition, parallel studies with murine cells may provide an answer to the question whether galactosylation or sialylation is the most likely immunomodulatory molecule candidate.

The data collected in the context of this thesis, in combination with literature findings, show a great potential for IgG-Fc glycosylation to play a role in the clinic when individuals are monitored over time. For other immunoglobulin glycosylation features, such as IgG-Fab or IgA, this has so far not been demonstrated.
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Rheumatoid arthritis (RA) is an autoimmune disease with a high prevalence in our society. The disease is known to reduce in severity (improve) during pregnancy. Adaptations in the immune system to prevent rejection of the fetus are thought to cause this improvement in RA patients. RA disease activity associates with a posttranslational modification on antibodies, namely N-glycosylation. Furthermore, N-glycosylation of immunoglobulin (Ig) G has been shown to change with pregnancy. Here, we developed and applied novel methodologies which allow for the detection of the glycosylation properties of Igs previously not or not properly detectable. By applying these methodologies on the PARA-cohort, a cohort designed to study the relation between RA and pregnancy, we tried to gain more insight into the association of glycosylation with the improvement of RA during pregnancy.

The first part of this thesis provides a general introduction to the topics discussed in the other parts. In Chapter 1 RA and pregnancy are introduced, with a focus on the combination of them, i.e., pregnancies of RA patients. Likewise, protein glycosylation is introduced. In the next chapter state-of-the-art immunoglobulin glycosylation analysis techniques are reviewed (Chapter 2). This provides the technological context of the thesis.

The second part of this thesis describes the development of novel techniques for broadening the scope of immunoglobulin glycosylation analyses. The relationship between RA disease activity and IgG glycosylation, more precisely the constant domain (Fc), is well-known. However, the variable antigen binding fragment (Fab) may also carry N-glycans. In Chapter 3 we describe a novel method which allowed, for the first time, higher-throughput Fab glycosylation analysis providing compositional information about the glycans. The method revealed distinct populations of N-glycans on the Fc and Fab regions of IgG. In addition, the method allowed detection of pregnancy-associated changes on the Fab, and in parallel show the known changes in Fc glycosylation.

Other Igs may also be involved in RA pathogenesis, as well as in the pregnancy-associated improvement thereof. These Igs are glycoproteins, as is IgG. Therefore we developed a method that would allow to study the glycosylation of IgA, which carries O-glycans in addition to N-glycans. The site-specific properties of N- as well as O-glycosylation of IgA1 could be analyzed in a higher throughput fashion by using the method described in Chapter 4. Several pregnancy-associated changes were revealed.

In the third part of this thesis the application of these and other novel technologies to a large clinical sample set is described. The samples were collected in the framework of the PARA-cohort (Pregnancy-induced Amelioration of Rheumatoid Arthritis; a large prospective cohort study on RA and pregnancy), which we studied in order to further our understanding of both the improvement of RA during pregnancy and the flare of disease activity observed after delivery. First, we applied a novel technique for the analysis of IgG Fc glycosylation. A lot of information was already available in this regard, but our technique allowed for improved detection of sialic acids, which were suggested to be most important in the suppression of inflammation. In Chapter 5 we confirmed the association of IgG-Fc glycosylation with disease activity. Furthermore we found that patients that improve, so-called ‘responders’,
showed different pregnancy-associated changes in their glycosylation compared to the non-responders. Similarly, the glycosylation changes of patients with a post-partum flare were found to be more pronounced compared to patients without a flare. Intriguingly, the suggested role of sialic acids appeared to be less relevant than expected, whereas in contrast galactosylation seemed to be most pronouncedly associated with disease activity. When we applied the technique described in Chapter 3 to the PARA-cohort, we found in Chapter 6 that the observed changes in Fab glycosylation were not associated with disease activity, and that healthy controls and RA patients hardly differed in their Fab glycosylation. For IgA glycosylation, studied on a large scale in Chapter 7, also in the RA patients some changes in glycosylation during pregnancy and after delivery were observed. However, these changes again appeared to be of limited value for explaining pregnancy-associated changes in disease activity, and no association with disease activity was observed. Finally, we looked into anti-citrullinated protein antibodies (ACPA) specific glycosylation. Autoantibody positive patients are less likely to improve during pregnancy. Therefore we examined whether the glycosylation of these RA-specific autoantibodies would shed some light on this phenomenon. Indeed, as described in Chapter 8, ACPA IgG Fc-glycosylation showed less pronounced pregnancy-related alterations, and did not associate with changes in disease activity. Interestingly, neither did total IgG in the ACPA positive patients, which is in contrast to ACPA negative patient findings. As is discussed in Chapter 9, this thesis gives new insights into the association of glycosylation with improvement of RA during pregnancy. More specifically, we showed that the glycosylation of IgG-Fc, in particular its increased galactosylation and not sialylation, is associated with decreased disease activity. In contrast, IgG-Fab glycosylation, IgA glycosylation, and ACPA-IgG glycosylation were found to hardly associate with disease activity. The unique association of total IgG-Fc galactosylation in relation to disease activity should still be further studied, but already suggests prognostic or predictive value. The glycans on the IgG-Fab portion are generally already highly galactosylated, and the galactose content therefore cannot be adapted so extensively. For these glycans the absolute number of glycans may have an effect, but this property has not been subjected to a sufficient amount of research to draw firm conclusions. Future research should provide more insight into this matter, and eventually solve whether aberrant glycosylation is either cause or effect of inflammation.
ADDENDUM II

NEDERLANDSE SAMENVATTING
Reumatoïde artritis (RA) is een auto-immuunziekte die veel voorkomt in onze samenleving. Het is bekend dat de ziekte verbetert tijdens de zwangerschap. Er wordt verondersteld dat deze ziekte-verbetering in de patiënten veroorzaakt wordt door aanpassingen aan het immuunsysteem die er voor moeten zorgen dat de foetus niet wordt afgestoten. RA ziekteactiviteit vertoont een samenhang met een post-translationele verandering van antilichamen, namelijk de N-glycosylering. Daarnaast is het aangetoond dat de N-glycosylering van immunoglobuline (Ig) G verandert met de zwangerschap. Wij hebben nieuwe methoden ontwikkeld en toegepast die het mogelijk maken om glycosyleringseigenschappen van Ig’s te detecteren die voorheen niet of niet goed te zien waren. Door het toepassen van deze methoden op het PARA-cohort, een cohort dat is ontworpen om de relatie tussen RA en zwangerschap te onderzoeken, hebben we geprobeerd om meer inzicht te krijgen in de samenhang tussen glycosylering en de verbetering van RA tijdens de zwangerschap.

Het eerste deel van deze thesis geeft een algemene introductie van de onderdelen die behandeld worden in de andere delen. In Hoofdstuk 1 worden RA en zwangerschap geïntroduceerd, in het bijzonder de combinatie tussen dezen, d.w.z. de zwangerschap van RA patiënten. Ook wordt eiwit glycosylering geïntroduceerd. In het daaropvolgende hoofdstuk worden de modernste immunoglobuline glycosylering analyse technieken besproken (Hoofdstuk 2). Dit geeft de technische context van deze thesis weer.

In het tweede deel van deze thesis wordt de ontwikkeling beschreven van twee nieuwe technieken die nieuwe mogelijkheden geven voor immunoglobuline glycosylering analyse. De associatie tussen RA ziekteactiviteit en IgG glycosylering, met name die van het constante deel (Fc), is bekend. Op het antigeen bindende fragment (Fab) kunnen echter ook N-glycanen voorkomen. In Hoofdstuk 3 beschrijven we een nieuwe methode voor de gedetailleerde compositionele analyse van Fab glycanen met een verhoogde doorvoersnelheid. De methode liet zien dat er verschillende populaties N-glycanen voorkomen op het Fc en Fab gedeelte van IgG. Daarnaast kon met de gebruikte methode worden aangetoond dat er rondom de zwangerschap ook op het Fab gedeelte veranderingen aan de glycanen optreden, terwijl deze tegelijkertijd voor het Fc gedeelte werden waargenomen.

Ook andere immunoglobulines zouden betrokken kunnen zijn bij de ontwikkeling van RA en de verbetering van RA tijdens de zwangerschap. Net als IgG zijn ook deze Ig’s geglycosylerde eiwitten. Daarom hebben we een methode ontwikkeld voor de glycosyleringsanalyse van IgA, die naast N-glycanen ook O-glycanen draagt. Deze eigenschappen per specifieke locatie binnen het eiwit konden met behulp van deze techniek met verhoogde doorvoer in Hoofdstuk 4 worden beschreven. Verscheidene veranderingen rondom de zwangerschap worden ontdekt.

In het derde deel van deze thesis wordt de toepassing van deze en andere nieuwe methoden op een grote verzameling klinische monsters beschreven. De monsters zijn verzameld binnen het kader van de PARA-studie (Pregnancy-induced Amelioration of Rheumatoid Arthritis; verbetering van RA met zwangerschap), met het doel om ons begrip van de verbetering van RA tijdens de zwangerschap en de verslechtering na de bevalling te vergroten. Allereerst
hebben we een vernieuwde techniek toegepast voor de analyse van IgG-Fc glycosylering. Veel hierover was al bekend, maar deze techniek maakte verbeterde detectie van siaalzuur mogelijk. Deze suikergroep werd verondersteld het meest belangrijk te zijn in de suppressie van ontsteking. In Hoofdstuk 5 bevestigden we de samenhang tussen IgG-Fc glycosylering met ziekteactiviteit. Daarnaast lieten we zien dat patiënten die verbeteren, de zogenaamde ‘responders’, andere veranderingen in glycosylering tijdens de zwangerschap laten zien dan patiënten die niet verbeteren. Een vergelijkbare observatie werd gedaan voor de verslechtering van de ziekteactiviteit na de bevalling, met een meer uitgesproken verandering in glycosylering in patiënten die verslechteren ten opzichte van patiënten die niet verslechteren. De eerdergenoemde rol van siaalzuur leek minder relevant te zijn dan verwacht, terwijl galactosylering daarentegen juist een meer uitgesproken associatie met ziekteactiviteit liet zien.

Bij het toepassen van de techniek die beschreven wordt in Hoofdstuk 3 op het PARA-cohort bleek dat de veranderingen die waargenomen werden op het Fab gedeelte niet samenhangen met de verandering in ziekteactiviteit, zoals beschreven in Hoofdstuk 6. Verder vonden we dat RA patiënten nauwelijks verschilden van gezonde controles wat betreft de Fab glycosylering.

De glycosylering van IgA in de monsters van het cohort, zoals beschreven in Hoofdstuk 7, laat ook voor de RA patiënten enkele veranderingen zien tijdens de zwangerschap en na de bevalling. Wederom lijken deze veranderingen in de glycosylering van weinig waarde als verklaring voor de verandering in ziekteactiviteit rondom de zwangerschap, aangezien er geen associatie tussen deze waarden werd gevonden.

Tenslotte hebben we de glycosylering van anti-gecitrullineerde eiwit antilichamen (ACPA) bestudeerd. Patiënten die auto-antilichamen hebben verbeteren minder vaak tijdens de zwangerschap. Daarom onderzochten we of de glycosylering van deze RA specifieke antilichamen hier een verklaring voor zou kunnen geven. En inderdaad, zoals beschreven in Hoofdstuk 8, de glycosylering van ACPA IgG-Fc veranderd minder met de zwangerschap, en laat geen samenhang zien met de ziekteactiviteit. Ook de glycosylering van totaal IgG-Fc in de patiënten die ACPA positief zijn heeft deze samenhang met ziekteactiviteit niet, terwijl dit wel het geval is in de ACPA negatieve patiënten.

Zoals bediscussieerd wordt in Hoofdstuk 9 geeft dit proefschrift nieuwe inzichten in de samenhang tussen glycosylering en de verbetering van RA tijdens de zwangerschap. Om precies te zijn hebben we laten zien dat de glycosylering van IgG-Fc, in het bijzonder de toename van galactosylering en niet die van siaalzylering, samenhang vertoont met een afname van ziekteactiviteit. Daarentegen laten IgG-Fab glycosylering, IgA glycosylering en ACPA IgG glycosylering deze samenhang niet of nauwelijks zien. Het unieke van totaal IgG-Fc galactosylering in relatie tot ziekteactiviteit dient nog verder onderzocht te worden, maar het geeft al uitzicht op mogelijke prognostische of voorspellende waarde. De glycanen op het variabele deel van IgG hebben over het algemeen al een hoge graad van galactosylering en er kan dus weinig galactose meer bij komen. Voor deze glycanen wordt verondersteld
dat wellicht het absolute aantal glycanen een effect heeft, maar deze eigenschap is nog niet voldoende onderzocht om daar krachtige uitspraken over te doen. Toekomstig onderzoek moet hier meer helderheid over gaan geven, en uiteindelijk de oplossing geven voor het vraagstuk of afwijkende glycosylering de oorzaak of het gevolg van ontsteking is.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAb</td>
<td>autoantibody</td>
</tr>
<tr>
<td>ACPA</td>
<td>anti-citrullinated protein antibodies</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CGE-LIF</td>
<td>capillary gel electrophoresis with laser-induced fluorescence detection</td>
</tr>
<tr>
<td>CH</td>
<td>conserved heavy chain</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAS28-3(CRP)</td>
<td>disease activity score based on three variables including CRP</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DMARDs</td>
<td>disease modifying anti-rheumatic drugs</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture dissociation</td>
</tr>
<tr>
<td>ERLIC</td>
<td>electrostatic repulsion HILIC</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>Fab</td>
<td>antigen binding fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystallizable</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-gamma receptor</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>Gal-0</td>
<td>non-galactosylated</td>
</tr>
<tr>
<td>Gal-1</td>
<td>monogalactosylated</td>
</tr>
<tr>
<td>Gal-2</td>
<td>digalactosylated</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GBP</td>
<td>glycan-binding protein</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>hinge region</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IgAN</td>
<td>IgA nephropathy</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IMGT</td>
<td>international ImMunoGeneTics information system</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulins</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose binding lectin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MQ</td>
<td>Milli-Q deionized water</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>PARA</td>
<td>Pregnancy-induced Amelioration of Rheumatoid Arthritis</td>
</tr>
<tr>
<td>PNGase F</td>
<td>peptide:N-glycosidase F</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>SA</td>
<td>sialic acid</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>Siglecs</td>
<td>Sialic acid-binding immunoglobulin type lectins</td>
</tr>
<tr>
<td>SNA</td>
<td>Sambucus nigra agglutinin</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>ZIC HILIC</td>
<td>zwitterionic HILIC</td>
</tr>
</tbody>
</table>
ADDENDUM IV

DANKWOORD
Dan is het ineens bijna klaar. Iets later dan van tevoren bedacht, maar het is er dan toch. En dan moet je gaan bedenken wie er allemaal heeft meegewerkt aan het tot stand komen van alles wat beschreven is, en van nog veel meer wat het niet heeft gered tot in dit ‘boekje’.

Het begint natuurlijk met de dagelijkse supervisie. Radboud, bedankt dat je me hielp om weer structuur te vinden als dat soms kwijt was. Manfred, bedankt dat je me de kans gaf om te groeien in de groep en de afdeling, en ook de ruimte bood om na de vier jaar nog even verder te gaan. En ook Mieke, bedankt voor de input die je hebt gegeven.

Verder kijkend zijn daar natuurlijk de mensen die de ruggengraat van elke research afdeling vormen, de analisten. In Rotterdam werd ik als dat nodig was fantastisch geholpen door Anne-Marie, Nadine en Patrick. En in Leiden natuurlijk door Agnes en Carolien, en ook Irina. De spaarzame momenten die ik in Rotterdam was waren altijd gezellig met Jan Piet, Sandra, Wendy en Odilia. Verder heb ik gezellig met Florentien gewerkt aan het opschonen van de PARA database, en met Martijn aan de statistiek voor een aantal manuscripten.

Op het LUMC heb ik een stuk meer tijd doorgebracht. Ik heb veel geleerd van, en veel lol gehad met, oud-collega’s zoals Maurice, Gerhild, Crina, Dennis, Dennis, en Yoann. Thanks for everything! Eigenlijk hoort Bas ook in het rijtje oud-collega’s, maar daarvoor hebben we nog teveel contact. De collega’s van nu zijn ook allemaal erg gezellig, waardoor het werken soms lastig is ;-). En ook nog behulpzaam. Vooral natuurlijk de directe aanspreekpunten, mijn (CPM) ‘roomies’ van T4-54: Karli, Noortje, Rosina, Kathrin, David en Martina. The same can be stated about our ‘neighbours’ who still don’t speak Dutch, despite the years they already spent here, Flo and Vika. Zoals het zo vaak het geval is op onze afdeling moeten we voor de volgende mensen naar een andere verdieping. Hier vinden we Glyco-mensen zoals Guinevere en Jan (c.s.), waarmee ik nog wat oude VHH-contacten heb opgesnord, wat vruchten af lijkt te werpen. Ook vind je op R3 Rico, die nooit te beroerd is om een vraag te beantwoorden, en Marco die de robot zijn kunstjes laat doen. Tegenwoordig moet je dan een brug over om terecht te komen bij een andere club erg gewaardeerde collega’s: Stephi, Bram, Hans en René. Na nog een paar kilometer kom je bij nog een groep huidige collega’s, waarvan vooral de samenwerking met Lise ook in deze thesis terug te vinden is, en nog steeds loopt.

Vergeet ik dan nog mensen in de werksfeer? Vast en zeker. Ik heb het er goed, of goed gehad. Dankzij veel mensen. Waarvoor dank!

Tijdens je tijd als PhD kan je jezelf verdrinken in je werk. Behalve als je een thuisfront hebt wat om aandacht vraagt. Wat een heerlijkheid om tijdens je promotie-traject te trouwen, en zelfs kinderen te krijgen! Dankjewel, Christine, voor je nooit aflatende steun. Dankjewel, Lukas, voor je inspiratie voor de cover. Dankjewel, Manuel, dat je lacht. Ook de verdere
familie en vrienden: dank voor steun, en voor interesse in dingen die vaak toch wel ver van je bed zijn.

En dank U, God, voor verstand, inzicht, energie en gezondheid om stukje bij beetje meer te begrijpen van wat U allemaal hebt bedacht.
Curriculum vitae
Albert Bondt was born on August 23rd 1984 in Deventer. He graduated from secondary education at Ichthys College in 2002. In February 2009 he obtained his Bachelor’s degree in Biology, and in December 2010 his Master’s degree in Molecular and Cellular Life Sciences. During his Master’s programme he followed internships at the Cellular Architecture and Dynamics group at Utrecht University, and at the MacInnes lab within the Hubrecht Institute in Utrecht. The last research project lead to his thesis entitled “Leucine functioning and branched-chain-amino-acid aminotransferase: Explaining the causes of Diamond-Blackfan anemia and cancer”.

In 2011 he started his PhD training at the department of Rheumatology at the Erasmus University Medical Center, under the supervision of dr. R.J.E.M. Dolhain and Prof. Dr. J.M.W. Hazes. In addition he spend a lot of time at the Center for Proteomics and Metabolomics (CPM; former Biomolecular Mass Spectrometry Unit) at Leiden University Medical Center (LUMC) under the supervision of Prof. Dr. M. Wuhrer.
Since 2015 he is working as a researcher at the department of Rheumatology and the Center for Proteomics and Metabolomics, both at the LUMC.
List of Publications

This thesis


Bondt A, Nicolardi S, Jansen BC, Kuijper TM, Hazes JMW, Van der Burgt YEM, Wuhrer M, Dolhain RJEM. IgA N- and O-glycosylation profiling reveals no association to the pregnancy-related improvement in rheumatoid arthritis. Submitted manuscript


Other


**PhD portfolio**

**Name PhD student:** Albert Bondt  
**Erasmus MC department:** Rheumatology  
**Research School:** Molecular Medicine  
**PhD period:** March 2011 – February 2015  
**Promotores:** Prof. Dr. J.M.W. Hazes and Prof. Dr. M. Wuhrer  
**PhD supervisor:** Dr. R.J.E.M. Dolhain

**Courses**
- ANAC Separation science  
- Working safe in laboratories  
- SmartTyping  
- Communication in Science  
- Laboratory animal science (Article 9)  
- Course on Molecular Medicine  
- CPM course Introduction to Glycobiology  
- Biomedical English Writing  
- Advanced Immunology

**(inter)national Scientific meetings**
- Joint Glycobiology Meeting Lille 2011 (oral)  
- MolMed Day 2012 (poster)  
- EWRR annual meeting Stockholm 2012 (poster)  
- Joint Glycobiology Meeting Wageningen 2012 (poster)  
- EULAR annual meeting Berlin 2012 (poster)  
- NVR Najaarsdagen 2012 (oral)  
- MolMed day 2013 (poster)  
- ACR annual meeting San Diego 2013 (poster)  
- HPLC conference Amsterdam (d.n.a.) 2013 (oral)  
- MolMed day 2014 (poster)  
- EWRR annual meeting Lisbon (d.n.a.) 2014 (poster)  
- Joint Glycobiology Meeting Gent 2014 (oral)

**Travel Grants**
- EULAR annual meeting Berlin

**Teaching**
- Supervised several bachelor students