Multifactorial aspects of antibody-mediated blood cell destruction
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Chapter 1

General introduction and scope of the thesis

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Antibodies in general

Immunoglobulins are crucial mediators of immunological protection against invading pathogens. Immunoglobulins are divided into 5 different isotypes, IgA, IgD, IgE, IgG and IgM, each with different functions and locations in the body. IgG is the most abundant antibody in lymph and blood, being divided in four subclasses, IgG1 (6.98 mg/ml in adult serum), IgG2 (3.8 mg/ml), IgG3 (0.51 mg/ml) and IgG4 (0.56 mg/ml). IgG1 and IG3 are the two subclasses mainly involved in Fc-receptor interactions and complement activation. IgG3 displays the strongest effector functions of all IgG-subclasses and is the main activator of the complement system, followed by IgG1. IgG3 has a half-life of only 7 days, while the other subclasses have a half-life of 21 days. The mutant H435-containing IgG3 (arginine at position 435 is exchanged with histidine) has a comparable half-life to IgG1.1 Immunoglobulins are comprised of 4 polypeptide chains, with two identical heavy chains and two identical light chains, assembled in a Y-form. The heavy chains of an IgG1 molecule weighs around 50 kD and has 5 isotypes, \( \gamma, \mu, \alpha, \varepsilon, \delta \). The light chain weighs around 25 kD and has two isoforms, \( \lambda \) and \( \kappa \), in total being approximately 150 kD for the whole immunoglobulin. Each arm of Y consists of a heavy and a light chain, while the stem of the Y consists of two heavy chains, all linked through disulfide bonds. The top of Y arm contains the variable region (Fab region), involved in antigen binding. The stem of the Y, thus the lower half of the light chain and all of the heavy chains are known as the constant region (Fc region). Despite being termed the Fc-region, this region can be considered to be variable as well due to the varying phenotype of the N-linked carbohydrate moiety attached at position 297. Antibodies can exert beneficial effects when directed against pathogens, but display an adverse effect when directed against for example platelets or red blood cells in auto- or alloimmune diseases.

Disease settings of antibody-mediated platelet and red blood cell destruction

Fetal or neonatal alloimmune thrombocytopenia (FNAIT)
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) develops when a pregnant woman, negative for a certain human platelet antigen (HPA) and carrying a fetus positive for that HPA, becomes immunized against the HPA antigen expressed by the fetus. Subsequently, the fetal platelets are targeted by the maternal anti-HPA IgG alloantibodies crossing the placenta. This leads to FcγR-mediated uptake of the sensitized fetal platelets by phagocytes in the fetal spleen and to a lesser extent in the liver, and may finally result in thrombocytopenia and increased bleeding tendency in the child.2 In Caucasians, these antibodies are mainly directed against HPA-1a, occurring in about 85% of FNAIT-cases. Immunization against
HPA-1a occurs in about 1:450 random pregnancies, resulting in FNAIT in 1:1200 and in severe FNAIT (<50 x 10^9 platelets/L) in 1:1800 pregnancies. Clinical outcomes may differ from asymptomatic (although perhaps thrombocytopenia may be present), to petechiae, or intracranial hemorrhage. In 10% of the cases with severe FNAIT an intracerebral haemorrhage can occur, and this complication might result in perinatal death or in severe neurological impairments. About half of these intracranial hemorrhages seem to occur before 28 gestational weeks and often affect the first born child.

Due to absence of routine screening for HPA-alloantibodies, the disease is only diagnosed after birth in symptomatic neonates, who show signs of an increased bleeding tendency, or otherwise it is discovered by chance. Therefore, currently only antenatal treatment is provided for pregnant women with a history of previously affected child, such as injections with high dose (0.5-2 g/Kg) intravenous immunoglobulins (IVIg); a treatment which may start already early from the second trimester until delivery, with or without corticosteroids. IVIg may compete with the placental transfer of pathogenic HPA alloantibodies, thereby lowering the anti-HPA antibody load in the fetus. It may also lead to relatively increased destruction of the anti-HPA alloantibodies in the mother. In addition other mechanisms, such as the presence of anti-idiotype antibodies in IVIg interfering with binding of the HPA alloantibodies to platelets in the child, but they may also interfere with the IgG Fc-receptor mediated sensitized platelet destruction. Despite the fact that the exact working-mechanism of IVIg remains to be elucidated, IVIg at 0.5 g to 2 g/Kg/week was shown to increase the platelet counts in most fetuses suffering from FNAIT. The repeated treatment with IVIg is in general well tolerated, but is costly and it has not been studied yet whether it has any influence on development of the child (for example of the immune system). Moreover, many anti-HPA1a-positive pregnancies will be needed to prevent a single severe case of ICH, since the majority of these children will not suffer from serious complications. As an alternative therapeutic monoclonal antibodies are being developed. Their proposed working-mechanism is to bind to fetal platelets, shielding them from the pathogenic anti-platelet alloantibodies and additionally preventing the fetal platelets from undergoing FcγR-mediated phagocytosis, thereby blocking platelet breakdown in the fetus or neonate. One of these antibodies is based on B2G1, a human anti-HPA-1a monoclonal antibody, of which the IgG1 residues of the Fc domain were substituted to abrogate binding ability to FcγRs. The placenta-crossing kinetics still need to be investigated, as well as clinical testing in randomized trials. Another therapeutic monoclonal antibody is based on the mouse anti-human platelet glycoprotein IIIa antibody SZ21, of which the Fc-portion has been deglycosylated preventing binding to FcγRs, yielding the NMG-SZ21 antibody. This antibody is able to cross the placenta but a humanized form of this antibody will be required for clinical trials. In either case, high doses of the therapeutic monoclonal antibody will probably be required considering the continuous exposure of maternal anti-platelet alloantibodies to fetal platelets as well as the possible HPA-
1a antigen-expression on endothelial cells\textsuperscript{10,11} and placental tissue.\textsuperscript{12,13} Other efforts are on its way in testing a pool of maternal plasma containing anti-HPA-1a antibodies as a prophylactic strategy, aimed at preventing HPA1a immunization in HPA1a-negative women carrying a HPA1a-positive child, in analogy to anti-D immunoprophylaxis.\textsuperscript{14,15} Many clinical studies are still warranted, including testing on pregnant women.

At present, no good predictive laboratory test exists to identify, among alloimmunized women with anti-platelet antibodies, those cases at risk of becoming thrombocytopenic with intracranial bleeding, unless treated. Therefore, and because the treatment of alloimmunized women to prevent bleeding in FNAIT cases has internationally not yet reached consensus, a general screening program for the detection of FNAIT has not yet been introduced. A large-scale Norwegian study has been conducted on more than 100,000 women which aimed to identify alloimmunized HPA-1a negative women and to offer them an intervention program, in order to reduce morbidity and mortality of FNAIT.\textsuperscript{16} This intervention program consisted of referring alloimmunized women to university hospitals for clinical follow-up. Delivery was performed by caesarean section 2-4 weeks prior to term, and HPA-1a negative platelets were available. Anti-HPA-1a antibodies were found in 10\% of the 2.1\% HPA-1a negative women. About 1/3 of the children were severely thrombocytopenic (<50*10^9/L), including two suffering from intracranial hemorrhages. Based on this large study, screening and intervention resulted in severe FNAIT-clinical complications in 3 of 57 cases (5.3\%). This was reduced in comparison to the 15 in previous prospective studies (136,814 women in total) which examined the frequency of FNAIT, and in those studies in total severe clinical complications occurred in 10 out 51 cases (19.6\%, with 3 intrauterine deaths and 7 intracranial hemorrhages).\textsuperscript{17-31}

**Immune thrombocytopenia (ITP)**

Similar to FNAIT, autoimmune thrombocytopenia (ITP) can also be an antibody-mediated disorder in which platelets are destroyed through activating IgG-Fc-receptors of phagocytes in the spleen and to a lesser extent in the liver, eventually resulting in thrombocytopenia. This was first discovered by Dr. William Harrington in 1951, when he infused himself with plasma from an ITP patient, causing his platelet count to drop dramatically within hours requiring hospitalization (Figure 1).

The plasma factor responsible for this effect was identified as immunoglobulin.\textsuperscript{32} Many decades later, an important role for T-cells in the pathogenesis of ITP was also recognized\textsuperscript{33-36} and platelets in ITP were also found to be destroyed by CD8-cytotoxic T lymphocytes (CTLs)\textsuperscript{37} and reactive oxygen species (ROS).\textsuperscript{38} ITP in childhood is characterized by a typical history of acute development of purpura and bruising in an otherwise healthy child, with an incidence of about 5 in 100,000 children.\textsuperscript{39} Most children with newly diagnosed ITP will not suffer from serious bleedings and will recover within three to six months. In some cases ITP becomes
chronic, and research indicates that chronic ITP may perhaps be a different disease entity than newly diagnosed ITP, based on serum cytokine levels and gene expression profiles.\textsuperscript{35,40-42} In about 60\% of ITP, there is a history of a prior infection.\textsuperscript{39,43,44} Initial management of newly diagnosed ITP may be either watchful waiting or pharmacologic intervention, such as glucocorticoid or IVIg. Second-line therapy includes dexamethasone, high-dose methylprednisolone, Rituximab and TPO-receptor agonists, next to combination regimens. Some ITP patients only respond to splenectomy, which is preferentially not performed at a young age.

**Hemolytic disease of the fetus and newborn (HDFN)**

Hemolytic disease of the fetus and newborn (HDFN) occurs when an antigen-negative pregnant woman becomes alloimmunized against a red blood cell antigen expressed by the red cells of her child. Red cell alloantibodies from IgG class cross the placenta and may destroy antigen-positive fetal red blood cells upon binding to FcγRs expressed by splenic macrophages. In the past the majority of HDFN cases were due to anti-RhD (anti-D) alloantibodies, but since the introduction of postnatal and later also antenatal anti-D immunoprophylaxis rates of RhD alloimmunisation decreased and allo-antibodies against Rhc and K have become equally important.\textsuperscript{45,46} In the Netherlands about 100 per 100,000 pregnancies are complicated by anti-D\textsuperscript{45}, whereas 191 per 100,000 pregnancies are at risk to develop HFDN because of another red cell antibody specificity (all pregnancies in which the father is typed positive for the involved antigen).\textsuperscript{47} Severe HDFN, in which intrapartum or postnatal exchange or...
blood transfusions were needed occurs in about 30% of anti-D complicated pregnancies, in 22% of anti-K complicated pregnancies (with K-positive child) and in about 11% of anti-c complicated pregnancies (with a c-positive child). Clinically, this can result in varying degree of anemia, hydrops, jaundice or even stillbirth. Jaundice is caused by an excess of bilirubin, which is released into the bloodstream during red blood cell destruction. Inability of the neonatal liver to conjugate and subsequently clear high levels of bilirubin can result in deposition in the skin or the brain, resulting in brain damage. Therapeutically, the removal of bilirubin can be stimulated by phototherapy or the concentrations of bilirubin can be lowered via exchange transfusions. In severe anemic fetuses also intra uterine transfusions are indicated to prevent severe disease. All pregnant women in the Netherlands are screened for anti-RBC-antibodies. When relevant anti-RBC antibodies are detected, laboratory testing is initiated using the antibody-titer as well as the monocyte-mediated antibody-dependent cell-mediated cytotoxicity (ADCC), a test measuring the cytolytic activity of antibodies in vitro. The monocyte-ADCC is more sensitive and specific to recognize fetuses at risk than the titer, as severe fetal anemia was shown not to occur in RhD-alloimmunized pregnancies in which the ADCC results remained <50%, while in cases with a maximum ADCC result of >80%, 43% of the fetuses were severely anemic, indicating that this test is extremely sensitive to indicate fetal anemia. If at increased risk, clinical monitoring is performed using Doppler flow measurement, which measures the blood flow in the arteria cerebri media, an indicator of a hyperdynamic circulation, typical for anemia (amongst other conditions). In contrast to other countries, which mostly rely on the titer for diagnostics, in the Netherlands the use of the ADCC test is recommended in the guideline from the Dutch Society of Obstetrics and Gynaecology to select patients for referral to the gynecologist and thereby preventing unnecessary referrals for women whose fetuses are not at risk to develop severe HDFN. Due to the success of the screening programming and subsequent timely interventions, extremely severe cases of HDFN are rarely reported in the Netherlands.

**IgG-Fc receptors**

Immunoglobulins exert their biological effects through several effector systems. For IgG-antibodies the most important effector functions are mediated through complement and/or the Fcγ-receptors (FcγRs), a family of cell surface receptors on leukocytes that specifically bind to the Fc portion of IgG-antibodies, bridging the adaptive and innate immune systems. In humans these receptors are termed FcγRIa (CD64a), FcγRIb (CD64b), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIC (CD32c), FcγRIIIa (CD16a), and FcγRIIIb (CD16b). With the exception of FcγRIIb, all the FcγR mediate activating functions (e.g. phagocytosis, ADCC, release of inflammatory mediators and superoxide radicals) after FcγR-crosslinking by IgG-
opsonized targets, while FcγRIIb mainly exhibits an inhibitory function, inhibiting the function of the activating FcγR. However, exceptions to this general rule has been found for anti-tumor responses through agonistic CD40 antibodies which requires FcγRIIb. These receptors also have a varying distribution on cells, with FcγRI mostly restricted to macrophages, monocytes and activated granulocytes, FcγRIIa having a widest range of expression on myeloid cells, FcγRIIIb only present on granulocytes (neutrophils, basophils and possibly on eosinophils) and FcγRIIIa on NK cells, macrophages and a subpopulation of monocytes, particularly in the spleen. Although it is clear that the inhibitory FcγRIIb is expressed on B cells and macrophages, the expression on other cells is less certain and may vary between individuals. An overview of the expression of FcγRs in different cell types in given in Table 1.

Table 1: Cellular distribution of FcγRs in human leukocytes.

<table>
<thead>
<tr>
<th>FcγR-type</th>
<th>FcγRI (CD64)</th>
<th>FcγRIIa (CD32a)</th>
<th>FcγRIIb (CD32b)</th>
<th>FcγRIIc (CD32c)</th>
<th>FcγRIIa (CD16a)</th>
<th>FcγRIIb (CD16b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular distribution</td>
<td>Macrophages</td>
<td>Macrophages</td>
<td>Macrophages</td>
<td>Macrophages</td>
<td>NK-cells*</td>
<td>NK-cells</td>
</tr>
<tr>
<td>human leukocytes</td>
<td>Monocytes</td>
<td>Monocytes</td>
<td>B cells</td>
<td>Monocytes</td>
<td>Basophils</td>
<td>Macrophages</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>Neutrophils</td>
<td>Basophils</td>
<td>Basophils</td>
<td>Platelets</td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>Mast cells</td>
<td></td>
<td></td>
<td></td>
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</table>

*Expressed on monocyte subset. # Expressed in 30% of humans. Uncertain distributions (and conflicting reports) have been omitted. Based on Hogarth et al.

Two other receptors for IgG are also expressed ubiquitously in almost all cells; the neonatal Fc-receptor (FcRn), a homologue of the major histocompatibility complex (MHC) class I molecules, and the tripartite motif-containing protein 21 (TRIM21), an E3 ubiquitin-protein ligase. Both receptors are expressed inside cells, particularly of myeloid origin. FcRn is found within vacuoles and tubules transporting FcRn along with its cargo, binding both endocytosed IgG and albumin in a pH-dependent manner, recycling both ligands and thereby extending their half-life. FcRn-mediated transport across cellular (e.g. epithelial, endothelial, syncytiotrophoblast) barriers is responsible for the IgG-transmission across mucosal barriers and from mother to child. In addition, FcRn participates in the process of IgG-mediated phagocytosis in myeloid cells in a pH-dependent manner independent of the IgG recycling. Downstream of this pathway FcRn can also deliver immune complex-bound antigens into the antigen presenting pathway, boosting secondary responses.

TRIM21, is expressed in the cytosol, binding IgG with an even higher affinity than the other human IgG-receptors. It recognizes opsonized non-enveloped viruses, intracellular bacteria and targets them for ubiquitination and proteasome degradation. Subsequently, TRIM21 activation has also been found to stimulate immune signaling via transcription factor pathways.
(NF-κB, AP-1, IRF3, IRF5, and IRF7), resulting in downstream secretion of pro-inflammatory cytokines, modulation of natural killer stress ligands and inducing an antiviral state\textsuperscript{62}. TRIM21 can therefore be considered as a cytosolic sensor for invading antibody-opsonized pathogens, and is therefore unlikely to play a role in immune-mediated cellular destruction.

Antibody-engagement through the surface-exposed FcγRs can also trigger intracellular signaling cascades (induced proximally and sequentially through Src- and Syk-kinases)\textsuperscript{63}. This can result in immediate degranulation of the cells, with concomitant release of inflammatory mediators and initiation of cellular responses, such as phagocytosis (by phagocytes such as monocytes, macrophages, neutrophils) or ADCC (for example by NK-cells or all myeloid cells). Under normal conditions these processes are utilized beneficially by the host to eliminate invading pathogens. However, these processes can also cause adverse reactions for the host in numerous auto-or alloimmune diseases\textsuperscript{64,65}, for instance by antibody-mediated platelet destruction (immune thrombocytopenia; ITP, or fetal or neonatal alloimmune thrombocytopenia; FNAIT)\textsuperscript{43} or antibody-mediated red blood cell destruction in hemolytic disease of the fetus or the newborn (HDFN).\textsuperscript{48}

Besides different cellular distribution of FcγRs, their affinity and specificity for the different IgG subclasses (IgG1, IgG2, IgG3, IgG4) also varies considerably, reflecting the distinct biological effects of each subclass in triggering different cell types (reviewed by Hogarth \textit{et al.}\textsuperscript{52}). For instance, IgG2 binds FcγRIIa and with slightly lower affinity for FcγRIIIa, while IgG1 and IgG3 bind all of the FcγRs. This enables IgG1 and IgG3 to co-cross link all FcγR (for instance FcγRIIa and FcγRIIib), while IgG2 targets only the FcγRIIa on neutrophils and possibly FcγRIIIa on NK cells, macrophages and some macrophages\textsuperscript{66}. The affinity for IgG differ greatly between FcγRI and the rest, but is also depending on the IgG subclass, as IgG1, IgG3 and IgG4 bind FcγRI in the nanomolar range, while IgG1 and IgG3 (and IgG4, but with slightly less affinity) bind FcγRII in the micromolar range.\textsuperscript{52,66} The binding affinities of the FcγRs with respect to IgG subclasses, are depicted in Table 2.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{FcγR-type} & \textbf{IgG-subclass affinity for human FcγRs} \\
\hline
FcγRI & IgG3 ≥ IgG1 > IgG4 > no binding IgG2 \\
FcγRIIa-H131 & IgG3 ≥ IgG1 = IgG2 > IgG4 \\
FcγRIIa-R131 & IgG3 ≥ IgG1 > IgG4 > IgG2 \\
FcγRIIb & IgG3 ≥ IgG1 ≥ IgG4 > no binding IgG2 \\
FcγRIlc & IgG3 ≥ IgG1 > IgG4 > no binding IgG2 \\
FcγRIIa-V158 & IgG3 > IgG1 > IgG4 > IgG2 \\
FcγRIIa-F158 & IgG3 > IgG1 > no binding IgG4 and IgG2 \\
FcγRIIib-NA-1/NA-2 & IgG3 ≥ IgG1 > no binding IgG4 and IgG2 \\
\hline
\end{tabular}
\caption{Overview of binding affinity of IgG-subclasses for human FcγRs.}
\end{table}

Highest IgG-subclass affinity for human FcγRs: IgG1, IgG3 and IgG4: highest affinity for FcγRI. IgG2: highest affinity for FcγRIIa-H131. FcγRIIa-V158 has a higher binding affinity for IgG1 and IgG3 compared to FcγRIIa-F158. Based on Hogarth \textit{et al.}\textsuperscript{52} and Bruhns \textit{et al.}\textsuperscript{66}
In last two decades we have become increasingly aware of the importance of how these binding affinities are affected by genetic polymorphisms within the FcγR family. A good example is FcγRIIa, which can have either arginine (R) or histidine (H) at position 131. The former binds human IgG2 with lower affinity than the latter, while the opposite is true for human IgG4 and mouse IgG1.52,66 Another important polymorphism in the FcγR-family can be found within FcγRIIIa, which contains either a valine (V) or phenylalanine (F) at position 158, with the V-variant showing stronger binding affinity for all the IgG subclasses.52,66-68 Consequently, the V-variant is associated with a higher incidence of ITP69,70, as well as with faster clearance of Rh D (RhD) expressing erythrocytes in the presence of anti-RhD IgG.71 Conversely, the lower affinity FcγR variants are associated with increased susceptibility to infectious diseases.72 For FcγRIIb, the inhibitory FcγR, a polymorphism exists within the trans-membrane region, with either an isoleucine (I) or threonine (T) at position 232 that determines their efficacy to co-aggregate with the activating FcγR into lipid rafts (T232 being less efficient) and thereby their capacity to down regulate the activating responses.73,74 An added layer of variability exists within the FcγR locus on the long arm of chromosome 1, which entails copy number polymorphisms of the FcγRIII genes as well as the FcγRIIc gene, which is a pseudogene in approximately 80% of healthy individuals. Those individuals expressing a functional copy of IIc have been found to be at increased risk for acquiring ITP (present in 34.4% of ITP patients), indicating that this polymorphism may also be an important negative predictor for autoimmune diseases.69 These polymorphisms and their importance for infectious diseases and auto- and allo- immunity have been extensively investigated and reviewed before.72,75-80 However, polymorphisms within the IgG, both endogenously encoded as well as through posttranscriptional modification, can also greatly affect immune responses, as will be described further below.

Ig-Fc glycosylation

IgG antibodies are glycoproteins containing a highly-conserved branched sugar moiety attached to the asparagine (ASN)297 part of the antibody-Fc domain. This glycan is essential for the maintenance of a functional structure and is required for binding of IgG with FcγR.81-83 Normally, this glycan consists of a core structures of N-acetylglucosamine and mannoses but with variable levels of galactose, sialic acid, bisecting N-acetylglucosamine (GlcNAc) and core fucose (Figure 2).

Glycosylation occurs in the lumen of the endoplasmatic reticulum (ER) and in the Golgi apparatus. The cellular glycan machinery in these organelles reflect the combined expression of several glycosyltransferases and glycosidases, of which there are over 200 in het mammalian genome.84,85 The formation and breakdown of glycans is complex and regulated at multiple
levels in the cell. The mechanism of cellular regulation of glycan expression include: 1) transcriptional regulation of genes encoding glycosyltransferases and glycosidases, 2) relative amounts of enzymes that compete for identical substrates, 3) intracellular enzyme trafficking and altered substrate accessibility, 4) synthesis and transport of nucleotide sugar donors to the ER and Golgi, 5) modulation of enzyme-structure through phosphorylation (based on preliminary data) and 6) intraluminal proteolysis in the Golgi resulting in secretion of catalytic domains (preliminary) and 7) glycan turnover at the cell surface by endocytosis and/or altered glycan synthesis. IgG-fucosylation can occur via two pathways which result in the formation of GDP-Fucose, the de novo synthesis and salvage pathways.86-89 De novo pathway occurs via GDP-mannose and GMD (GDP mannose dehydratase) and the salvage pathways occurs via extracellular L-fucose, which in the cytosol interacts with fucokinase. GDP-Fuc is then imported from the cytosol into the Golgi via GDP-Fucose transporters.88 GDP-Fucose is then the substrate for fucosyltransferase, fucosyltransferase 8 (FUT8) in the case of IgG, that then adds core fucose in the medial golgi to the IgG-Fc glycan at position ASN297.

Interactions between various elements of IgG-glycosylation have been reported. As is clear from Figure 2, lack of galactose residues will result in a concomitant absence of terminal sialic acid.90 However, it has been found that the amount found of non-sialylated bigalactosylated IgGl correlated negatively with the degree of disialylated glycans.90,91 This indicates that bigalactosylated glycans are a better a substrate for sialyltransferases than monogalactosylated glycans, converting bigalactosylated glycans quickly into monosialylated and disialylated digalactosylated structures. A surprising finding was also that a large increase in the proportion of bisecting GlcNAc was observed to contain disialylated structures.91 In addition, fucosylation and bisection can show negative correlations, as reported for glyco-engineering of therapeutic antibodies by exchanging the localization domain of beta1,4-N-acetylglucosaminyltransferase III (GnT-III) with that of other Golgi-resident enzymes.
demonstrating that GnT-III can compete even more efficiently against the endogenous core alpha1,6-fucosyltransferase (alpha1,6-FucT) and Golgi alpha-mannosidase II (ManII) leading to higher proportions of bisected non-fucosylated glycans. However, this artificial localization of GnT-III lead to this almost complete blockage of fucosylation. Additionally, this correlation was also reported in another study. Bisecton was also shown to be negatively correlated to galactosylation.

Not much is known about the regulation of Fc-glycosylation. Interestingly, B-cell stimuli have been shown to modulate Fc glycosylation. Both the TLR9 ligand CpG oligodeoxynucleotide and IL-21 have been shown to increase Fc galactosylation and reduce bisecting GlcNAc levels. In contrast, all-trans retinoic acid decreases galactosylation and sialylation levels. Also T-cell independent B cell activation has been shown to induce immunosuppressive sialylated IgG antibodies in mouse models. Recently, the transcription factor Hepatocyte Nuclear Factor (HNF)1a and its downstream target HNF4a were described to be transcriptional regulators of key fucosyltransferases and fucose biosynthesis genes. Furthermore, it was shown that variation in 51 out of 76 IgG glycan traits studied was at least half heritable, with only a small proportion of N-glycan traits having a low genetic contribution. Some of the highly heritable glycans were shown to be affected by multiple loci, amongst them FG0n/G0n (the percentage of fucosylation, without bisecting GlcNAc, of agalactosylated structures), which associated with genetic variants in: FUT8, MGAT3, IKZF1 and SMARCB1-DERL3. More research is required to elucidate the regulatory pathways involved in IgG-glycosylation.

Variation in this composition influences antibody affinity to FcγR and thus antibody effector activity. Increased sialic acid content of IgG-Fc has received a great amount of attention over the past years due to their protective effect in IVIG. First, sialic acid-containing IgG was demonstrated to have an approximate 7-fold reduced binding affinity to murine IgG to FcγRIII in mice, preventing antibody-mediated platelet-depletion in vivo. Sialic acid-containing IgG in IVIG has been described to enable IgG-binding to the mouse lectin SIGN-R1 (in humans known as dendritic cell-specific ICAM3-grabbing non-integrin - or DC-SIGN), up-regulating a cytokine cascade initiated by IL-33 and eventually leading to enhanced myeloid expression of FcγRIIb via IL-4, which suppressed serum-induced arthritis in mice. Supposedly, the sialic acid was attached to the Fc-part of the IgG, however, two other groups have shown that the Sambucus nigra agglutinin-lectin based enrichment protocol used to obtain the IgG in some of these studies, preferentially enriches Fab-containing sialic acid IgG and has no effect on immunomodulating activity of IVIg. The protective effect of IgG-sialic acid has been recently challenged by a murine study of Rheumatoid Arthritis, a disease well known for frequent remission during pregnancy. Although this remission seemed to go hand in hand with both increased galactosylation and sialylation, a further independent analysis of di-galactosylated (G2) IgG indicated that the association with sialylation to be an epiphenomenon due to its requirement for previous galactosylation. In fact, while G2- IgG
was associated with remission, disialylated G2-IgG had an opposing effect apparently by the minor but positive association with disease activity.\textsuperscript{103}

Of the variable amount of glycans present in the IgG-Fc, the removal of the IgG-core fucose has by far the greatest effect on binding to FcγRs. A lack of core fucose has been demonstrated to result in an up to 50-fold stronger binding affinity to FcγRIIIa \textsuperscript{81,104} and FcγRIIIb \textsuperscript{104} but not to the other FcγRs. This restriction is caused by a conserved N-glycan in human FcγRIIIa and FcγRIIIb at position 162 (N162), which interacts with the Fc but also the Fc-glycan. This configuration is greatly affected by the presence or absence of the core-fucose within the IgG-Fc.\textsuperscript{83,105} The enhanced binding to the GPI-linked FcγRIIIb has been shown to result in enhanced neutrophil phagocytosis of rituximab-opsonized CD20\textsuperscript{+} lymphoma cells.\textsuperscript{104} In addition, the enhanced binding to FcγRIIIa resulted in enhanced ADCC mediated by mononuclear cells \textsuperscript{81,106-110}, but remarkably not through neutrophil FcγRIIIb.\textsuperscript{111} Perhaps this can be explained if neutrophil-mediated ADCC occurs more via the ITAM of FcγRIIa than through the GPI-linked FcγRIIIb with both receptor types boosting phagocytosis.

The importance of the core fucosylation of the IgG-Fc has only received attention because of the possibilities to produce more efficacious therapeutic antibodies, like rituximab (reviewed by Yamane-Ohnuki et al).\textsuperscript{112} This is because core fucosylation was generally not found to be to be very variable in humans. Indeed, \textasciitilde94\% of all naturally occurring IgG-derived Fc-glycopeptides in humans have the core-fucose attached. Theoretically, this means that \textasciitilde99.7\% of all IgG contain either one (combination of a heavy chain without and with fucose on either side or 2 x 0.06 x 0.94 * 100\%= 11.3\%) or two (0.94 x 0.94 * 100\%=88.4\%) core-Fc fucoses, with only 0.3\% of circulating IgG completely devoid of core-fucose. However individual differences do exist, for instance neutral IgG glycans lacking core fucose were found to vary between 1.3\% and 19\%.\textsuperscript{91} In addition, this was recently exemplified in a genome-wide association study, identifying the loci containing FUT8 and the transcription regulator Ikaros to influence these levels – loci that in turn were previously reported to be associated with other diseases such as systemic lupus erythematosus, Crohn\'s disease, and multiple sclerosis.\textsuperscript{113} Theoretically, antigen-specific IgG-fucosylation could be skewed in certain immune responses (compared to total IgG). The remarkable variation in IgG-Fc glycosylation patterns in various disease settings, including autoimmune diseases and several types of cancer, and their subsequent effects reported in the literature are summarized in Table 3.

At the start of this thesis almost no studies on the glycosylation pattern of antigen-specific IgG were performed, and therefore most changes in these diseases apply to total IgG. Since then only few papers on antigen-specific IgG glycosylation have appeared, amongst them regarding Lambert-Eaton myasthenic syndrome, in which the IgG1 directed against anti-presynaptic volt-gated Ca\textsuperscript{2+}-channels demonstrated a decreased galactosylation and for patients below 50 years of age an increased bisection.\textsuperscript{124}
In this thesis we will mainly focus on antibody-mediated diseases in pregnancy (FNAIT and HDFN). In that respect IgG glycosylation patterns in pregnancy are also of importance. In pregnancy, total IgG-galactosylation and – sialylation are found to be increased, while – bisection decreased.\textsuperscript{114-116}

Regarding gender and age, galactosylation and sialylation were found to be increased in females at young age and decreased for both males and females with increasing age.\textsuperscript{91,126,127} The most potent decrease in levels of galactosylation and sialylation in females, was around the menopause (age 45-60).\textsuperscript{127} Age-related glycosylation changes were more pronounced in individuals <57 years compared to individuals >57, and more pronounced in females than in males.\textsuperscript{127} Bisection was increased in younger individuals and came to a plateau at older age.\textsuperscript{127}

### Table 3: Overview of IgG-glycosylation patterns and their effects in disease-settings.

<table>
<thead>
<tr>
<th>IgG-glycan</th>
<th>Disease setting</th>
<th>Affected-IgG</th>
<th>Effect of IgG-glycan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosylation</td>
<td>Rheumatoid Arthritis (RA)</td>
<td>Total IgG</td>
<td>Increased\textsuperscript{114-116}, increased in remission of RA in pregnancy\textsuperscript{103,114,115,117}, decreased during relapse of RA in pregnancy\textsuperscript{117}, inversely related with N-acetylglucosamine\textsuperscript{118,119}</td>
</tr>
<tr>
<td></td>
<td>Juvenile onset chronic arthritis</td>
<td>Total IgG</td>
<td>Inversely related with N-acetylglucosamine\textsuperscript{118,119}</td>
</tr>
<tr>
<td></td>
<td>Crohn’s disease</td>
<td>Total IgG</td>
<td>Inversely related with N-acetylglucosamine\textsuperscript{119}</td>
</tr>
<tr>
<td></td>
<td>Primary osteoarthritis</td>
<td>Total IgG</td>
<td>Decreased\textsuperscript{120}</td>
</tr>
<tr>
<td></td>
<td>Osteoarthritis</td>
<td>Total IgG</td>
<td>Parallel increase with with N-acetylglucosamine\textsuperscript{119}</td>
</tr>
<tr>
<td></td>
<td>Sjögren’s Syndrome</td>
<td>Total IgG</td>
<td>Decreased\textsuperscript{118}. Parallel increase with with N-acetylglucosamine\textsuperscript{119}</td>
</tr>
<tr>
<td></td>
<td>Tuberculosis</td>
<td>Total IgG</td>
<td>Decreased\textsuperscript{121}</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Total IgG</td>
<td>Decreased\textsuperscript{122}</td>
</tr>
<tr>
<td></td>
<td>Gastric cancer</td>
<td>Total IgG</td>
<td>Decreased\textsuperscript{123}</td>
</tr>
<tr>
<td></td>
<td>Lambert-Eaton myasthenic syndrome</td>
<td>IgG1-anti presynaptic voltage gated Ca\textsuperscript{2+}-channels</td>
<td>Decreased\textsuperscript{124}</td>
</tr>
</tbody>
</table>

| Sialylation     | RA                                   | Total IgG    | Increased in remission of RA in pregnancy\textsuperscript{103,115}                  |
|                | Gastric cancer                       | Total IgG    | Decreased\textsuperscript{123}                                                    |

| Fucose      | Gastric cancer (stage II-III)         | Total IgG    | Increased\textsuperscript{123}                                                    |
|            | Infertile leukospermic patients       | Seminal IgG  | Decreased compared to normal, fertile normozoospermic patients\textsuperscript{125} |

| Bisecting N-acetyl-glucosamine | Gastric cancer (stage II-III)         | Total IgG    | Decreased\textsuperscript{123}                                                    |
|                                | Lambert-Eaton myasthenic syndrome (<50 years) | IgG1-anti presynaptic voltage gated Ca\textsuperscript{2+}-channels | Increased\textsuperscript{124}                                                    |
Looking at female sex hormones, estradiol was associated with IgG1 glycans, showing a decreased G0F, increased agalactosylation, decreased G0FN, increased G2F, increased digalactosylation, increased galactosylation and increased G2.\textsuperscript{128} Digalactosylated glycoforms correlated significantly higher with female sex hormones than monogalactosylated glycoforms. In the same study, no associations were found for afucosylation and sialylation as regards to estradiol and progesterone. The association of IgG glycans with progesterone was lessened compared to estradiol. However, progesterone was shown to activate the oligosaccharyltransferase complex in the lumen of the endoplasmatic reticulum, resulting in increased IgG-N glycosylation. In addition, it was shown that IL-6 and progesterone could modulate the asymmetric glycosylation of Fabs.\textsuperscript{129-131}

In summary, Fc-coupled glycan structures are important for antibody-mediated immune responses, and it is also apparent that glycosylation profiles are tightly regulated by physiological, pathological and genetic factors.

In conclusion, several variables including FcγR polymorphisms, IgG titer and subclass, IgG-glycosylation affect IgG-effector functions and clinical outcomes. Strong effector functions and high binding affinities can be associated with a “good” outcome in terms of protection against infectious diseases or antibody-mediated therapies. However, these can also turn out “bad” or even “ugly” in allo- and auto-immune mediated diseases. All these factors should be taken into consideration when looking into the biological response, both from a diagnostic as well as a therapeutic perspective.

**Scope of the thesis**

The research described in this thesis focuses on diseases of antibody-mediated platelet destruction, in particular on FNAIT, and diseases of antibody-mediated red blood cell destruction, such as HDFN, in order to better understand the pathogenesis and clinical consequences of the diseases. Diagnostically, for HDFN laboratory tests are in place in order to predict risk for severe fetal RBC destruction and thereby initiate appropriate treatments. This test is sensitive, but has relatively low specificity. For FNAIT, no diagnostic laboratory test is currently available to predict severe fetal platelet destruction. For FNAIT it has been suggested that the decrease in platelet numbers is determined by the antibody titer, so the higher the antibody-titer, the lower the platelet counts.\textsuperscript{132-134} Also standardized MAIPA protocols were developed for the quantification of anti-HPA-1a antibodies.\textsuperscript{135} However, this correlation does not appear to be that strict, due to the presence of cases with low antibody titers which still caused severe platelet destruction (Figure 3).
This indicates that other factors, besides antibody-titer, are involved. We analyzed in Chapter 2 if we could set up and optimize sensitive functional in vitro assays measuring antibody mediated platelet phagocytosis and phagocytic-respiratory burst. We then aimed to utilize these assays to identify possible novel factors, besides the antibody titer, involved in antibody-mediated platelet destruction, and to investigate their working-mechanism, and if they are elevated in patient sera and to correlate to platelet counts and clinical disease severity.

FNAIT patients can be either asymptomatic or petechiae can occur, but also major organ bleedings or the most severe complication of intracranial hemorrhage can occur. As the titer is not strictly related to disease severity, we first wondered if the FNAIT disease severity might be influenced by glycosylation pattern of platelet allo-antibodies, so that laboratory monitoring of glycosylation patterns might be applied to identify high-risk cases with potent anti-HPA1a-Fc-receptor interaction. We therefore first analyzed antibody-glycosylation in a healthy pregnancy setting, comparing the glycosylation profile of total IgG between pairs of mother and fetus (Chapter 3), to test if these would differ from each other, as for IgG-glycosylation measurements (FNAIT and HDFN) maternal serum samples will be utilized, but the destruction is directed against the fetus. Earlier reports have described significant differences in total glycosylation between fetal and maternal IgG, suggesting alternative maternal transport of IgG, as FcRn binding to IgG is not dependent on Fc-glycosylation. Whereas these early studies were performed by releasing N-glycans from total IgG, we chose for analysis of Fc-glycosylation at the glycopeptides level, in a Fc-and subclass specific manner, looking at galactosylation, sialylation, bisecting GlcNAc and fucosylation.

Hereafter, we aimed to isolate these anti-platelet alloantibodies from sera, followed by subsequent characterization of the Fc-glycosylation patterns, in a pilot study featuring 8 sera with allo-antibodies against different platelet antigens from different diseases (FNAIT post-transfusion purpura and refractory thrombocytopenia) (Chapter 4). Then we aimed
to optimize our antibody-isolation technique from serum, if needed, and expand on anti-HPA-1a antibody samples (~50 samples) for the purpose of evaluating glycosylation patterns of maternal platelet-alloantibodies and its relation with phagocyte effector functions and disease severity in FNAIT (Chapter 5). In the same chapter, we additionally questioned how the FNAIT glycosylation patterns would be compared to another disease of antibody-mediated platelet destruction, such as refractory thrombocytopenia, in which anti-HLA class I antibodies are frequently implicated. This could shed light on the regulation of the observed glycosylation patterns in the sense of pregnancy vs non-pregnancy.

Similar to FNAIT for platelets, HDFN can occur for RBCs and as a comparison we aimed to investigate the Fc-glycosylation patterns of anti-D antibodies of pregnant women. In this way we could compare the glycosylation profiles of both diseases, as both of them are from a pregnancy setting, in both the target is blood cell, but the antigenic target is different. As for HDFN already tests for recognizing high risk patients are utilized in diagnostics (titer and monocyte-mediated ADCC), with high sensitivity but relatively low specificity, we also aimed to relate the glycosylation profiles to those tests and clinically to hemoglobin values, to see if glycosylation affects RBC destruction and thus monitoring could be of added value in the prevention of HDFN (Chapter 6).

In Chapter 7 we expand on anti-D glycosylation in pregnancy, by investigating anti-D Fc-glycosylation from hyperimmunized male and female donors, from which anti-D immunoprophylactic products are generated and used to prevent immunizations in pregnancy. Is the glycosylation profile of male hyperimmununized donors similar that of pregnant women and female hyperimmununized donors? This well shed light on the requirement of a pregnancy setting for the observed glycosylation profile. In addition, we aim to investigate several commercial immunoprophylactic preparation as regards to anti-D glycosylation, based on the hypothesis that anti-D Fc-glycosylation may be an important factor in the working-mechanism of anti-D immunoprophylaxis. Differences in anti-D glycosylation could therefore unmask variation in biological activity (despite the titer). Also the hyperimmunized anti-D (HID)-donor immunization history will be analyzed as regards to the glycosylation profile, as this could possibly lead to improvement in HID-donor selection and thus in the composition of the final immunoprophylactic product.

The key-findings will finally be discussed in Chapter 8.
References


126. Ruhaak LR, Uh HW, Beekman M et al. Decreased levels of bisecting GlcNAc glycoforms of IgG are associated with human longevity. *PLoS.One.* 2010;5(9):e12566.


