Multifactorial aspects of antibody-mediated blood cell destruction
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Low but highly variable Fc-fucosylation of anti-D IgG1 in pregnancy

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Key points

1. Anti-D alloantibodies causing HDFN have decreased but highly variable Fc fucosylation.
2. Decreased Fc-fucose correlates with enhanced FcγRIIIa-mediated ADCC, as well as with more severe hemolysis.

Abstract

Hemolytic disease of the fetus and newborn (HFDN) may occur when maternal anti-D IgG antibodies cross the placenta and mediate the destruction of D-positive red blood cells (RBCs) via phagocytic-IgG Fc-receptors (FcγR). Clinical severity is not strictly related to titer, but is more accurately predicted by functional antibody-dependent cellular cytotoxicity (ADCC), suggesting other factors to play a role. Since the composition of the N-linked glycan at position 297 in the Fc-region of IgG1 is critical for binding to FcγR, we analyzed IgG-derived glyco-peptides from anti-D IgG1 purified from plasma of alloimmunized pregnant women by mass spectrometry. Seventy pregnancy-induced anti-D samples, taken from different women, revealed a varied, but strong decrease in Fc-fucosylation in the majority of anti-D IgG1, but not in total IgG. The degree of anti-D fucosylation correlated significantly with CD16 (FcγRIIIa)-mediated antibody-dependent cellular cytotoxicity (ADCC) by NK-cells, in agreement with the increased affinity of defucosylated IgG to human FcγRIII. In addition, the degree of anti-D fucosylation correlated significantly with fetal-neonatal hemoglobin levels, suggesting IgG-fucosylation to be an important pathological feature in HFDN.
Introduction

Hemolytic disease of the fetus or newborn (HFDN) arises due to maternal alloimmunization against paternally inherited fetal red blood cell antigens, most commonly Rh-D. These anti-D alloantibodies are transported across the placenta, bind to D-positive fetal red blood cells (RBCs) and subsequently engage with phagocytic-IgG Fc-receptors (FcγR), resulting in red blood cell clearance. Clinically, this can result in fetal anemia, jaundice, hydrops and stillbirth.\(^1\) The administration of anti-D immunoprophylaxis to women at risk has greatly reduced the immunization rate.\(^2\) Furthermore, the occurrence of severe fetal/neonatal complications that despite prophylaxis occur in alloimmunized women is prevented in most developed countries thanks to nationwide red cell antibody screening programs.\(^3\) Once an alloantibody is recognized, the pregnant women are carefully monitored to start timely treatment. We have previously shown that the most sensitive laboratory test to predict fetal red cell destruction is the ADCC assay;\(^4\) and for that reason in the Netherlands all pregnant women with red blood cell alloantibodies are monitored using this assay and are only referred for Doppler flow measurement, when the ADCC assay is above a certain threshold. This suggests that the interaction of the antibody with phagocytic cells is an important factor determining the risk of the disease. The strength of the interaction between IgG and FcγR, and hence the strength of the phagocyte response, depends on several factors, including the IgG subclass involved, the FcγR polymorphic make up of the patient, but also on the IgG-Fc glycosylation.\(^5\)

IgG antibodies are glycoproteins harboring a branched sugar moiety attached to asparagine (Asn) 297 in the Fc domain. The glycan is important for the functional structure and is required for binding of IgG to FcγR. Furthermore, slight variations in this glycan composition modulate the affinity.\(^6-8\) Besides an invariant structure composed of N-acetylgalactosamines (GlcNac) and mannoses present in this Asn297-linked glycan, galactose, sialic acid, bisecting N-acetylgalactosamine (GlcNac) and fucose can be either absent or present. The relative abundance of these glycans has been found to be altered for total IgG in various clinical settings, including pregnancy,\(^9-11\) cancer,\(^12,13\) and autoimmunity\(^9-11,14-19\) and infectious diseases.\(^20\) For most of these glycan modifications, the reported changes for FcγR binding are modest, however, the lack of core fucose results in much stronger binding to human FcγRIII, due to glycan-glycan interactions between Asn297 in IgG1 and Asn162 found only in FcγRIIIa and FcγRIIIb.\(^8,21,22\)

We have recently shown that anti-human platelet antigen (HPA) 1a IgG1 antibodies, formed during pregnancy against HPA-1a positive fetal platelets, can display a pronounced decrease in Fc-fucose, resulting in an increased binding affinity for FcγRIIIa/b, an enhanced platelet-phagocytosis and a more adverse fetal or neonatal alloimmune thrombocytopenia (FNAIT).\(^23\) Here we tested if similar low Fc-fucosylation can also be found in pregnancy-induced anti-D allo-IgG1 antibodies, and if skewed anti-D IgG1 fucosylation may explain the discrepancy between anti-D titer and clinical severity, which is observed in some cases.
Methods

Patient samples
Anti-D alloantibodies were diagnosed at Sanquin, Amsterdam, Netherlands (n=70) using the indirect antiglobulin test with the addition of PEG and the use of a polyclonal anti-IgG (Sanquin reagents, Amsterdam). The titer was determined with a two-fold dilution in a tube indirect antiglobulin test (no additions, incubation 30 minutes 37°C, 3 washings, polyclonal anti-IgG and monoclonal anti-C3d) against RBCs with a R2R2 (ccDEE) phenotype. Clinical data was obtained retrospectively by contacting obstetric care givers. Samples were obtained with informed consent from the patients in accordance with the Declaration of Helsinki.

Purification and IgG quantification of anti-D antibodies from sera
Anti-D alloantibodies were purified from serum by incubation of the serum with D-positive RBC (500 µl serum for anti-D titer of 256 or lower, and for titer of 512, 250 µl of serum was used, all incubated with 500 µl packed D-positive RBC), for 1 hour at 37°C. Similarly, D-negative RBC incubated with D-positive serum, as well as D-positive RBC incubated with Normal Human Serum (NHS), were used as negative controls. This was followed by 6 washings with cold PBS. Hereafter, antibodies were eluted from the RBC via addition of eluting buffer (low-pH glycine buffer of Gamma ELU-KIT™ II, Immucor, Inc., Norcross, GA 30071, USA) and the pH of the antibody-containing eluate (supernatant) was neutralized by addition of 250 µL of basic Tris-phosphate buffer (214 mM Tris, 22 mM Na₂HPO₄). The specificity of the eluate was confirmed by gelscards (DiaMed GmbH, Bio-Rad Laboratories, Inc., Hercules, CA, USA, 6 microtubes containing rabbit anti-human IgG within the gel matrix). The amount of IgG1 and IgG3 in the eluate was determined by ELISA on solid plates (NUNC-Immuno, Maxisorp, Sigma Aldrich, St Louis, MO, USA) coated with either mouse-anti-human IgG1 Fc (clone MH161-1, Sanquin reagents, Amsterdam, The Netherlands) or with mouse-anti-human IgG3 hinge (clone MH163-1, Sanquin reagents, Amsterdam, The Netherlands). For calculations of IgG1 and IgG3 concentrations, fully human recombinant antibodies (IgG1κ and IgG3 GDob124) were used as standard. Each eluate was only considered to contain purified anti-D IgG if all negative controls tested below threshold, and test samples contained IgG with a positive reaction to D-positive cells only.

IgG Purification from eluates
IgG1 was isolated from plasma and eluates by subclass specific affinity chromatography using 10 µL CaptureSelect™ IgG1 (Hu) affinity beads in 96-well plate format 0.7 ml/well filter plates (Orochem, Naperville, IL). Serum (2 µL) or affinity-purified anti-D antibodies (200-4000 ng as determined by ELISA) were applied to the affinity beads in 200 µL of PBS, followed by a 1 h incubation at RT with shaking. Then the IgG1 affinity beads were washed by filtration with 3
times 200 μL PBS and with 3 times 200 μL water. Bound IgG1 was eluted with 100 mM formic acid (100 μL; Fluka, Steinheim, Germany) and dried by vacuum centrifugation. The purified IgG1 was digested overnight at 37°C with 200 ng trypsin (Promega, Leiden, The Netherlands) in 40 μL 25 mM ammonium bicarbonate buffer (Fluka). Samples were stored at -20°C until further use.

Reverse-Phase Solid Phase Extraction (RP-SPE) of Glycopeptides
IgG1 Fc glycopeptides were enriched and desalted by reverse phase (RP) solid phase extraction (SPE) using Chromabond C18ec (C-18) beads (Marcherey-Nagel, Düren, Germany). The C-18 beads were activated by 80% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA; Fluka) and conditioned with 0.1% TFA. Tryptic IgG1 digests (40 μL) were added to 150 μL 0.1% TFA, loaded onto C-18 beads, followed by 3 washes with 100 μL 0.1% TFA. Elution of the IgG glycopeptides were performed by 18% ACN containing 0.1% TFA to minimize coelution of interfering peptides. The purified glycopeptides were dried by vacuum centrifugation and stored at -20°C until mass spectrometric analysis.

MALDI-TOF-MS of IgG Glycopeptides
Analysis of RP-SPE purified IgG1 Fc-glycopeptides was performed using MALDI-TOF-MS. Samples were dissolved in 40 μL of water, and 2 μL aliquots were spotted onto MTP 384 polished steel target plates (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Subsequently, 2 μL of 5 mg/mL 4-chloro-α-cyanocinnamic acid (Cl-CCA; 95% purity; Bionet Research, Camelford, Cornwall, U.K.) in 70% ACN was applied on top of each sample and allowed to dry. Glycopeptides were analyzed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics), which was operated in the positive-ion reflectron mode. Ions between m/z 1000 and 3700 were recorded. To allow homogeneous spot sampling, a random walk laser movement with 100 laser shots per raster spot was applied, and each IgG glycopeptide sum mass spectrum was generated by accumulation of 2000 laser shots. Mass spectra were internally calibrated using a list of known glycopeptides (G0, G0F, G1, G1F, G2, G2F species, see Table 1).

Data processing and evaluation were performed with FlexAnalysis Software (Bruker Daltonics) and Microsoft Excel, respectively. The data were baseline subtracted and the intensities of a defined set of 12 glycopeptides (6 glycoforms for IgG1 with core fucose and 6 glycoforms for IgG1 without core fucose) were automatically defined for each spectrum.

Relative intensities of IgG Fc glycopeptides were obtained by integrating the first isotopic peaks followed by normalization to the total IgG1 specific glycopeptide intensities. The level of galactosylation was calculated from the relative intensities of various Fc N-glycopeptides according to the formula: (G1 + G1F+ G1FN + G1N)*0.5 + G2 + G2F + G2N + G2FN+G2NS. The prevalence of bisecting GlcNAc was determined by summing the relative intensities of
all bisected glycoforms (G0N, G0FN, G1N, G1FN, G2N and G2FN). The incidence of IgG1 fucosylation was evaluated by summing all fucosylated IgG1 Fc N-glycoforms (G0F + G1F + G0FN + G2F + G1FN + G2FN).

**NK-cell and monocyte based antibody-dependent cellular cytotoxicity (ADCC)**

D-positive RBCs were labeled with radioactive chromium 51 ($^{51}$Cr) and incubated with maternal serum containing anti-D antibodies at an equal titer of 128. Peripheral blood mononuclear cells were isolated from blood taken from healthy volunteers, via density gradient centrifugation over ficoll (GE Healthcare, Uppsala, Sweden) 1.077 g/ml) and platelets were removed by washing. Monocytes were depleted by adherence to plastic tissue culture flasks for 1 hour at 37°C, after which the non-adherent fraction was taken and allowed to adhere to plastic tissue culture flasks again for 1 hour at 37°C. The non-adherent cells were washed and re-suspended in RPMI with 20% human AB serum and incubated for 1.5 hours at 37°C in triplicate in U-well microplates with $^{51}$Cr-labeled D-positive RBCs (3*10^6/ml, 150,000 per well), in a total volume of 100 µl and an effector to target cell ratio of 15:1. Maximum lysis was obtained with the addition of 150 µl 5% saponine to 50 µl of RBCs and was used to calculate the degree of lysis (%). Similarly, the monocyte-ADCC was performed (as described previously)^4, with 1.5*10^6/ml monocytes (75,000 per well and derived from a pool of 70-100 donors, stored in liquid nitrogen). Cytotoxic lysis was assessed by counting the $^{51}$Cr-activity in the supernatant and by expressing it as a percentage of lysis produced by a pooled polyclonal anti-D standard according to a calibration curve.

**Results**

**Fc-glycosylation of anti-D IgG1 in pregnancy**

Total IgG and anti-D alloantibodies were purified from 70 maternal pregnancy-anti-D sera via acidic elution from D-positive RBCs, and tryptic IgG-derived peptides encompassing the Asn297-Fc glycan were subsequently analyzed by mass spectrometry (MALDI-TOF-MS). The quantity of 12 most abundant IgG1-Fc glycopeptides were examined (Table 1).

For total IgG1, the Fc glycosylation profile was abundant in fucosylated glycan structures (G0F, G1F, G2F, Fig 1A), as described for normal human IgG1.\(^{11,23,26,27}\) In contrast, the anti-D specific IgG1-derived glycopeptides mostly consisted of glycoforms lacking core fucose (G1 and G2, Fig. 1B).

Systematic analysis of the 70 pregnancy-induced anti-D IgG1 sera for Fc-galactosylation, -bisection (bisecting GlcNAc) and -fucosylation, showed that the levels of Fc-galactosylation were significantly increased (Fig. 2A) and the levels of Fc-bisection significantly decreased (Fig. 2B), when compared to total IgG1 (galactosylation: 61.92% and bisection 13.80% in...
non-pregnancy setting). Strikingly, the levels of Fc-fucosylation were found to be variably decreased, with some cases with levels of core fucosylation down to 10%, while this was fairly constant for total IgG1 (on average 93.3% ± 4.6, Fig. 2C), which is comparable to the total IgG1-fucosylation in a non-pregnancy setting (92.86%). Furthermore, we observed a weak but significant negative correlation between anti-D IgG1-fucosylation and galactosylation ($R^2$:0.06. P=0.023) and no significant correlation between bisection and galactosylation or bisection and fucosylation (Supplementary Fig. 1).

**Fc-fucosylation levels of pregnancy-induced anti-D IgG1 predict NK-cell mediated ADCC activity.**

As monocyte ADCC is used to monitor anti-D activity for diagnostic purposes, since it is more predictive for disease severity of HDFN than the antibody titer, we first investigated if glycosylation of the anti-D IgG1 affected the biological activity of the anti-D. A very weak, albeit significant, correlation between monocyte ADCC and the level of galactosylation was found (Fig. 3A). No significant correlation was found between monocyte ADCC and bisection or fucosylation. However, as IgG-Fc fucosylation only affects binding to FcγRIIIa which

### Table 1 Theoretical masses of tryptic glycopeptides of human IgG1.

<table>
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<tr>
<th>Glycan species</th>
<th>Glycan structure</th>
<th>IgG1 E$<em>{293}$EQYNSTYR$</em>{301}$ $^{a}$ P01857$^{b}$ [M+H]$^{+}$</th>
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Glycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F) and bisecting N-acetylglucosamine (N). $^{a}$Tryptic IgG glycopeptide sequence. $^{b}$Uniprot entry number
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**Figure 1** IgG1 glycosylation profiles of a pregnant woman with anti-D.

Tryptic IgG1 Fc glycopeptides of total IgG1 (A) and anti-D specific IgG1 (B) affinity-purified from serum of a pregnant woman, were measured by positive-ion reflectron mode MALDI-TOF-MS. G0, agalactosylated glycan without core fucose; G0F, agalactosylated glycan with core fucose; G1, monogalactosylated glycan without core fucose; G1F, monogalactosylated glycan with core fucose; G2, monogalactosylated glycan without core fucose; G2F, monogalactosylated glycan with core fucose. Blue square, N-acetylglucosamine; green circle, mannose; yellow circle, galactose; red triangle, fucose; pep, peptide moiety; *, contaminant. For the assignment of the glycopeptides signals see Table 1.

**Figure 2** Anti-D IgG1 in pregnancy display a pronounced lowered Fc-fucosylation.

Relative expression levels of major IgG-Fc Asn-297 glycoforms for both total IgG1 (x-axis) and antigen-specific IgG1 (y-axis) for 70 pregnancy-induced anti-D serum samples (A-C). Serum populations were analyzed for Fc-galactosylation (A), Fc-bisection (bisecting N-acetylglucosamine) (B) and Fc-fucosylation (C). The statistical outcome between two-tailed paired t-test analysis of total IgG1 Vs. specific antibodies is listed in each panel. The diagonal, dotted line represents theoretical equal ratio between total IgG1 and anti-D IgG1. Normal total IgG1 values (non-pregnancy setting): galactose (61.92%), bisection (13.80%) and fucose (92.86%)\(^1\)
is hardly expressed on circulating monocytes (~5% of cells show low expression), we also tested the functional capacity of some of these pregnancy-induced anti-D IgG1 antibodies to mediate RBC lysis through CD16+ (FcyRIIIa) through NK cells-mediated ADCC. For eleven samples sufficient material was available to perform this assay. With this set of samples we did not observe any significant correlation between anti-D IgG1 galactosylation or bisection and NK-cell ADCC (Fig. 3D-E, respectively), and also not with monocyte ADCC (Supplementary Fig. 2). However, in line with the strong FcγRIIIa expression on NK cells the degree of anti-D IgG1 fucosylation correlated significantly with NK-cell mediated ADCC, with a lower degree of anti-D Fc-fucosylation corresponding to an increased NK-cell mediated ADCC (Fig. 3F).

Figure 3 Low anti-D IgG1-fucosylation induces stronger NK-cell mediated ADCC, but not monocyte-ADCC.

Monocyte ADCC (A-C), and NK cell ADCC (D-F) versus the levels of galactosylation (A, D), bisection (B, E) and fucosylation (C, D) found in IgG1-anti-D. For the monocyte-ADCC the data for all samples were plotted, but only those containing enough material and with anti-D titer of 1/128 for the NK-cell mediated ADCC. Statistical analyses were performed by two-tailed (A-E) and one-tailed (F) Pearson correlation.

Low fucosylation of IgG1 anti-D is associated with low neonatal hemoglobin levels at birth

IgG-opsonized RBCs are cleared quickly through FcγR-receptor mediated pathways in the liver and spleen.28 We therefore, as a pilot-study, tested if the hemoglobin levels in the fetus or neonate were associated with IgG1-anti-D fucosylation in these patients. All patients
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were included with dominantly IgG1-anti-D and of which the hemoglobin levels, before any IUT, were recorded (n=12). In agreement with lowered anti-D IgG1 core fucosylation and its increased affinity to FcγRIIIa, we found the degree of IgG1-anti-D Fc-fucosylation to correlate significantly with the hemoglobin levels (Fig. 4, $R^2=0.289$ and $P: *, 0.037$).

**Discussion**

In the current paper we investigated the IgG1-Fc-glycosylation patterns of 70 pregnancy-induced anti-D antibodies. The glycosylation pattern of the anti-D specific IgG was compared to that of total IgG from the same patient. The antigen-specific IgG1-antibodies were first affinity purified from D-positive RBCs, and then using anti-IgG1 beads, after which trypsin digested glycopeptides were analyzed by mass spectrometry. Despite the presence of IgG3 anti-D in some of the patients, we now focused solely on IgG1-Fc glycosylation in pregnancy. IgG3-derived glycopeptide detection by mass spectrometry is complex due to the presence of multiple IgG3 allotypes in the population, that have either identical mass to that of IgG2 or IgG4 glycopeptides. In general, the anti-D IgG1 antibodies displayed elevated levels of galactosylation, lowered bisection, but most prominently, a decrease in core fucosylation was observed in many of the patients. Importantly, the affinity purification procedure using protein G Sepharose beads with acidic elution did not result in a noticeable change of IgG Fc glycosylation profiles. This was assessed by comparing the glycosylation profile of a highly core-fucosylated monoclonal antibody before and after protein G affinity purification (not shown). Besides, the changes observed were reproducible upon re-measuring, and highly variable between patients, with some patients showing no changes in anti-D glycosylation patterns.
Recently, we described a similar decrease of IgG1-Fc fucosylation in anti-HPA-1a IgG1 antibodies in FNAIT, which we did not observe for anti-HLA antibodies in platelet transfusion refractoriness (refractory thrombocytopenia, RT).\textsuperscript{23} Moreover, we found this decreased Fc-fucosylation to result in enhanced platelet-phagocytosis mediated by FcγRIIIa-positive monocytes or FcγRIIIb-positive PMN. Similarly, this kind of skewing of core fucosylation in antigen-specific IgG has now also been described in one recent study on anti-HIV responses in elite controllers of HIV infection but unlike in our studies, these also displayed agalactosylated glycans.\textsuperscript{30}

Besides a decrease in fucosylation of the anti-D-specific IgG1, we also found a decrease in bisecting GlcNac compared to total IgG, similar to what we found for anti-HLA antibodies in a cohort of patients who received platelet transfusions.\textsuperscript{23} However, this lowered level of bisection was not found in FNAIT-anti-HPA-1a IgG1,\textsuperscript{23} perhaps indicating a differentiation of the B cell responses depending on the context of the target antigen. Bisection was neither a predictor of NK nor monocyte ADCC activity towards red cells.

We also observed an increased Fc-galactosylation of both total IgG and more pronounced for D-specific IgG. This was not unexpected, as total IgG-galactosylation is known to be increased in pregnancy,\textsuperscript{9-11} and because we previously found that decrease in core fucosylation goes hand-in-hand with increased galactosylation in anti-HPA-1a IgG1 found in FNAIT.\textsuperscript{23} However, there does not seem to be a direct causal relationship between the level of fucosylation and galactosylation in general, as decreased fucosylation and galactosylation has been observed in anti-HIV IgG1.\textsuperscript{30}

Due to the fact we measured the samples in the mass spectrometer in positive-ion mode for increased sensitivity, we were unfortunately not able to obtain reliable data for sialylation. However, based on our previous analysis of patient’s anti-HPA1a antibodies, and \textit{in vitro} generated antibodies, where we have found that increased galactosylation generally results in increased sialylation since the substrate for sialyltransferases adding α2-6 sialic acid are β1,4 galactose residues,\textsuperscript{31} a similar increase in sialylation of the anti-D specific IgG1 is to be expected. This fits with a recent report showing 16% sialylation of anti-D specific IgG compared to 8% for total IgG.\textsuperscript{32} Most importantly, addition of galactose to the ASN297 glycan in IgG has been shown to result in decreased binding to FcyR\textsuperscript{33} which is further reduced by addition of sialic acid.\textsuperscript{34} This is supported by a recent report showing that lack of galactosylation enhances the pathogenic activity of IgG1 anti-RBC autoantibodies.\textsuperscript{35} Together this makes it is very unlikely that the increased galactosylation we observe, with or without possible increased sialylation, will positively affect ADCC. Although the degree of galactosylation did correlate significantly with monocyte ADCC, the affect was marginal at best, with no such correlation being noted for the NK-cell mediated ADCC. However, we cannot exclude that a possible effect might be masked by the concomitant decrease in fucosylation that would be expected to have the opposite effect due the increased binding to FcyRIII. Similarly to the findings in
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In this small group of patients there was no significant correlation between hemoglobin levels at birth and anti-D titer. However, in a clinical setting in alloimmunized women, a rise in titer is generally accompanied by a rise in ADCC. Although the anti-D titer is a relevant parameter for the prediction of fetal red cell destruction,\(^1\) the monocyte-mediated ADCC has been shown to be a better predictor;\(^4\) as it also takes the interaction of the antibody with the phagocytic FcγRs into account, even though the FcγRIIIa-negative peripheral monocytes are probably not strongly participating in RBC removal \textit{in vivo}, unlike the FcγRIIIa-positive counterparts found in the spleen.\(^{28}\) The monocyte-based ADCC is extremely sensitive, as shown by the fact that severe fetal anemia did not occur when the ADCC results remain <50\%.\(^4\) Conversely, when ADCC level were high (>80\%), 43\% of the fetuses were severely anemic.\(^4\) The specificity of the ADCC test for prediction of severe HDFN is also higher compared than measurement titer measurement,\(^1\) but in terms of prediction of severe anaemia the specificity is relatively low. The results of the present study provide a possible explanation, as we now show an individual variation in the levels of low-core fucose among the anti-D antibodies, which predicts higher affinity to FcRIIIa, which is not detected by the monocyte
ADCC. Monitoring the degree of IgG Fc-fucosylation, either directly or by an NK cell ADCC, might improve the specificity of the laboratory screening for detecting fetuses at high risk. In agreement with this, we found a positive correlation between the hemoglobin levels at birth in relation and IgG1-anti-D Fc fucosylation in pregnancy.

In conclusion, the IgG-glycosylation we find in pregnancy responses against RhD bear remarkable similarities to what we have previously reported for anti-platelet responses. This holds true, in particular, with the potent lowering of core fucosylation of the antigen-specific IgG. In addition, the lowered core Fc-fucose in anti-D IgG1 was associated with increased FcγRIIIa-mediated ADCC and decreased hemoglobin levels at birth, suggesting this type of glycosylation to be an important biomarker and therapeutic target in HDFN.

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Authorship Contributions

R.K. performed all purifications of anti-D alloantibodies from serum, confirmed the specificity of the eluate by geldcards, co-ordinated the ADCC experiments and analyzed clinical data; L.D.V. gathered serum samples together with P.L., determined the amount of IgG1 and IgG3 in the eluate via ELISA; M.S. gathered clinical data; A.H.E. prepared samples, conducted the mass spectrometry and processed the raw mass spectrometry data with M.W., R.V. isolated cells for the ADCC, P.L. provided cells for antibody purifications from serum; R.K., G.V., M.W. made the figures and tables, R.K. performed statistical analysis, G.V. conceived the study and G.V., C.E.v.d.S. and M.W. supervised the study; M.d.H. co-supervised the study; R.K., G.V, C.E.v.d.S., M.W. wrote the paper, which was critically revised and approved by all authors, and all authors contributed to analysis and interpretation of data.

Conflict-of-Interest Disclosure

The authors declare no competing financial interests.
Supporting information

Supplemental figures

**Supplementary Figure 1** The relationships between the degree of glycosylation of anti-D IgG1 for A) galactosylation and fucosylation, B) galactosylation and bisection, and (C) fucosylation and bisection. Significance was tested by two-tailed Pearson correlation, and significance was set at 0.05. NS: non-significant.

**Supplementary Figure 2** Monocyte-mediated ADCC towards RBC vs glycosylation of anti-D IgG1 using the same set of samples as in Fig. 3D-F), shown for A) galacosylation, B) bisection, C) fucosylation. Significance was tested by two-tailed Pearson correlation. Significance was set at 0.05. NS: non-significant.
References


32. Winkler A, Berger M, Ehlers M. Anti-rhesus D prophylaxis in pregnant women is based on sialylated IgG antibodies. *F1000Res.* 2013:2


