Hemolytic disease of the fetus and newborn: awareness precedes change

Winter, D.P. de

PART V

INNOVATIONS TO IMPROVE DIAGNOSTICS AND MANAGEMENT


CHAPTER
10

IgG-Fc-Glycosylation And A Novel Flowcytometric Assay To Predict Hemolytic Disease Of The Fetus And Newborn

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ABSTRACT

Introduction: Timely and accurate referral of pregnancies with red blood cell (RBC) alloantibodies is essential to prevent perinatal death or long-term adverse consequences in hemolytic disease of the fetus and newborn (HDFN). Classically, antibody titers are used to select high-risk pregnancies. Research suggested that also IgG-Fc-fucosylation could be an important pathological feature. We evaluated the diagnostic potential of IgG-Fc-fucosylation in identifying high-risk pregnancies, and we report on a flowcytometric assay that integrates antibody quantity, antibody subclass and fetal FcγRIIIa affinity.

Methods: Maternal serum samples from a nationwide prospective cohort of D-alloimmunized pregnancies, between 2014 and 2017, were used to evaluate IgG-Fc-glycosylation in mass-spectrometry (n=64), and the flowcytometric assay (n=90). Results were compared to standard-of-care titers and antibody-dependent cellular-cytotoxicity assay (ADCC) results. Receiver operating curves (ROC) were used to assess and quantify predictive values.

Results: The cohort for mass-spectrometry analysis consisted of 31 pregnancies without transfusions (48%), 16 with IUT (25%) and 17 (27%) with postnatal transfusions only. The flowcytometric assay consisted of 53 (59%) pregnancies without transfusions, 17 (19%) pregnancies with IUT, and 20 (22%) pregnancies with postnatal transfusions only, suggesting that severe HDFN was overrepresented. Anti-D IgG1 and IgG3-Fc-fucosylation levels were lower in groups requiring transfusions, but no distinct difference between groups was detected. No significant difference in other glycan traits was found. Among sera from 90 D-alloimmunized pregnancies evaluated in the flowcytometric assay, we found significant differences in IgG- and FcγRIIIa binding between pregnancies without transfusions and with IUT, suggesting a potential predictive value for the need for IUTs. ROC analyses revealed a lower false-positive rate at a 100% sensitivity in the prediction of pregnancies at-risk for fetal anemia through the flowcytometric assay (20.8% for IgG binding and 43.1% for FcγRIIIa binding) compared to the titer and ADCC (74.6%).

Conclusion: Anti-D IgG-Fc-fucosylation levels tended to be lower in pregnancies with IUT, but could not distinguish pregnancies with or without IUT in this cohort. The flowcytometric assay, measuring IgG levels and functional FcγRIIIa binding, indicates that the specificity of serological monitoring in HDFN may be improved. Considering an overrepresentation of severe HDFN in this cohort, we advise to confirm these findings in a prospective cohort within a routine setting.

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is a life-threatening disease in which fetal and neonatal red blood cells (RBC) are hemolyzed due to maternal alloantibodies against paternally inherited blood group antigens, most commonly RhD.[1,2] Throughout pregnancy, IgG antibodies are actively transported across the placenta where they may opsonize fetal RBCs. These opsonized RBCs, which in case of anti-D do not fix complement[3], are recognized by Fc-gamma receptors (FcγR) expressed on phagocytes, leading to hemolysis and clearance. Binding of antibodies to FcγRs, and thereby the phagocytic response, is dependent on multiple factors, including IgG subclass, IgG-Fc-glycosylation and fetal FcγR polymorphisms.[3-8]

All IgG antibodies have a highly conserved N-glycosylation site at position 297 in the CH2-domain of the Fc-region. The attached glycan is crucial for the Fc-tail's quaternary structure and stability, but also influences effector functions and, thereby, the clinical progression of immune responses. It contains a bi-antennary core-structure, which comprises multiple N-acetylglucosamine (GlcNAc) and mannose residues. This core-structure can be extended with additional fucose, bisecting GlcNAc, up to two galactose, and up to sialic acid residues. Although in healthy individuals the glycosylation profile is relatively stable with little intra-individual variation, we have previously demonstrated that the glycosylation profile of anti-D is vastly different from total IgG with high intra-individual variation.[7] Anti-D shows decreased levels of core fucosylation and increased levels of galactosylation.[7,9] The absence of fucose improves the binding affinity to FcγRIIIa/b up to 20 fold, or even 40 fold if also highly galactosylated.[3,10] These differences may lead to even larger functional effects for induced effector functions, including Antibody-Dependent Cellular-Cytotoxicity (ADCC) and -Phagocytosis (ADCP)[3,5,10,11], and ultimately disease severity.[7,11,12] Anti-D fucosylation levels correlated with FcγRIIIa-mediated NK-cell ADCC, and with fetal or neonatal hemoglobin levels, suggesting that IgG-Fc-fucosylation could be an important pathological feature in HDFN.[3,5,7]

Antenatally, alloantibodies to RBC antigens, including anti-D, may cause fetal anemia. If fetal anemia is not timely detected through ultrasound and treated with intrauterine transfusions (IUT), this may develop into hydrops fetalis and ultimately perinatal death.[2] Postnatally, hyperbilirubinemia and anemia may occur, that may be treated with intensive phototherapy, exchange transfusions or simple transfusions.[2] The prevalence of anti-D-mediated HDFN has decreased significantly in high-income countries after the implementation anti-D immunoprophylaxis (RhIG) to prevent RhD-alloimmunization.[13,14] Additionally, timely intervention in alloimmunized pregnancies have reduced severe complications.[15,16]

In the Netherlands, repeat serological monitoring with the antibody titer and ADCC assay is performed in alloimmunized pregnancies to identify those at risk of fetal anemia who subsequently require ultrasound monitoring. The monocyte-ADCC assay, a functional biological assay used to indicate the pathogenicity of the antibody, improved diagnostic accuracy compared to solely antibody titers.[17] However, despite its strong predictive value, there are drawbacks to the routine use of the monocyte-ADCC as it is labor-intensive, costly, requires pooled monocytes isolated from a large number of volunteer donors, and in some laboratories, including ours, is still dependent on radioactive material. Thus, there is need for a simplified and strong predictive diagnostic assay in HDFN.

In this study, we aimed to evaluate the diagnostic potential of IgG-Fc-fucosylation to accurately identify pregnancies with severe D-mediated HDFN. We additionally tested and report upon a novel flowcytometric assay in which binding of FcγRIIIa-V158, the subtype with highest affinity, and level of IgG opsonization are determined. We aimed to compare the clinical value of the flowcytometric assay for the prediction of severe HDFN to the standard maternal antibody titer and monocyte-ADCC-assay.

METHODS

Human Subjects

Maternal serum samples were collected in the OPZI 2.0 prospective cohort, a nationwide study on D-alloimmunization during pregnancy, as described previously.[18] Briefly, pregnant women were invited to participate between 2014 and 2017 when tested positive for anti-D alloantibodies, determined at Sanquin Diagnostic Services. Written informed consent was retrieved by obstetric care providers. Samples from included pregnancies were collected from leftover material remaining from serological monitoring, described below. In the current study, samples were selected under the condition that the anti-D titer differed no more than one dilution step from the maximum titer. Samples prior to the first IUT were selected in pregnancies with IUT.

Monitoring and treatment of D-alloimmunized pregnancies

In the Netherlands all pregnancies are screened for the presence of RBC alloantibodies in the first trimester. If clinically relevant alloantibodies are found and the fetus is positive for the implicated antigen, determined through cell-free fetal DNA in maternal plasma, then these pregnancies are sequentially serologically monitored by determination of the antibody titer and ADCC. For D-alloimmunization specifically, pregnancies with

a titer of 1:16 or above and/or an ADCC reaching or exceeding 50% are regarded as at-risk for fetal anemia and are consequently referred to the Leiden University Medical Center (LUMC) for sonographic monitoring to timely detect and treat fetal anemia. The LUMC is the national referral center for fetal therapy. Sonographic monitoring is done through weekly Doppler measurements of the fetal peak-systolic velocity of the middle cerebral artery (MCA-PSV) and assessments of secondary signs of anemia (e.g. cardiomegaly, hepatosplenomegaly, reduced fetal movements). An IUT is performed if anemia is suspected, based on an MCA-PSV ≥1.5 multiple of the median and presence of secondary signs.[19,20]

Purification of anti-D alloantibodies from sera

To assess glycosylation traits of anti-D IgG1 and IgG2/3 alloantibodies, we purified samples based on methods previously described.[7] In short, 500 µl serum of included cases was incubated with 100 µl packed D-positive RBC (phenotype [C-c+D+E+e-; R2R2; double-dose D-antigens)] for 1 hour at 37 °C. Followed by eight washes with 12 mL cold phosphate-buffered saline (PBS). Antibodies were then eluted by low-pH glycine buffer of Gamma ELU-KIT™. Then, 250 µl Tris-PBS was used to neutralize the pH. MoAb anti-D incubated with R2R2 phenotype RBC's were used as positive controls. As negative controls, Group AB serum without RBC antibodies - was incubated with R2R2 phenotype or rr phenotype RBC's (C-c+D-E-e+); and MoAb anti-D was incubated with rr phenotype RBCs. Elutes were stored at -80 °C until mass spectrometry.

IgG-Fc-glycosylation analysis by mass spectrometry

Total IgG was enriched from 1 µl serum using CaptureSelect™ FcXL Affinity beads ((Thermo Scientific), followed by tryptic digestion to generate IgG Fc glycopeptides as previously described.[21] For cell eluates, total IgG was affinity-purified from 300 µl neutralized eluates using FcXL beads,100 mM FA solution was used for antibody elution, followed by sample drying by vacuum centrifugation. The purified antibodies were dissolved in 20 µL reduction-alkylation buffer (50 mM ammonium bicarbonate, 10 mM tris(2-arboxyethyl)phosphine (TCEP) and 40 mM chloroacetamide), incubated for 30 min at 60 °C, followed by overnight tryptic digestion at 37 °C. Fore cleanup, 5mg Chromabond C18 endcapped beads (45 µm; Macherey-Nagel, Düren, Germany) were added to a filter plate (Orochem) and washed three times with 200 µL 80% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA), followed by three washes with 200 µL 0.1% TFA. Digests were diluted in 0.1%TFA to a total volume of 400 µL and added to the beads, followed by 3 min shaking at 600 rpm. Beads were washed 3x with 200 µL 0.1% TFA. The enriched glycopeptides were eluted using 100 µL 18% ACN/0.1% TFA, then

dried by vacuum centrifugation and reconstituted with MQ. IgG Fc glycopeptides were separated and detected by an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA) coupled with an Impact quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA), as described previously[21], IgGs were identified based on accurate mass and specific retention times in liquid chromatography.

LC-MS data processing

LaCyTools[22], an in-house developed software was used for the alignment and targeted extraction of the mzXML files converted from the raw LC-MS spectra. Alignment was performed based on the average retention time of at least three highly abundant glycoforms. The analyte list for targeted extraction of the 2+ and 3+ charge states was based on manual annotation and literature reports. Inclusion of an analyte in the final analyte list was based on quality criteria including signal-to-noise (>6), isotopic pattern quality (<25% deviation from the theoretical isotopic pattern), and mass error (±20 parts per million range) (Supplemental Table 1). The relative intensity of each glycan species in the final analyte list was calculated by normalizing to the sum of their total areas. Spectra were excluded if their sum intensity was below the average sum intensity of the anti-D IgG signal of negative controls. Normalized intensities were used to calculate fucosylation, bisection, galactosylation, and sialylation (Supplemental Table 2).

Production and glycoengineering of anti-D monoclonal antibodies

In the development of a flowcytometric assay to predict disease severity using FcγRIIIa binding levels and total IgG and IgG1/IgG3 subclass specific opsonization levels, we produced and glycoengineered anti-D MoAbs (Clone 19A10) in a Human Embryonic Kidney (HEK) 293F production system, as described in detail before.[3,6,23] In short, transfection vectors (pEE6.4 or pEE14.4) were designed and cloned, encoding either hIgG1 HC + anti-D VH, hIgG3 HC + anti-D VH or Kappa LC with anti-D VL.[3,24] In order to produce afucosylated MoAbs, 0.4 mM of the chemical inhibitor 2-deoxy-2-fluoro-L-fucose (2FF, Carbosynth) was added 4 hours post-transfection. The MoAbs were harvested and purified six days after transfection, using the ÄKTA Prime Plus (GE HealthCare) equipped with protein A HiTrap HP column (GE HealthCare). The MoAbs were subjected to liquid chromatography – mass spectrometry based IgG-Fc-glycosylation analysis to verify the desired fucosylation levels.[23,25]

Recombinant biotinylated FcγRIIIa-V158

FcγRIIIa-V158 monomers were produced and c-terminally biotinylated as described previously.[26-28] Briefly, pcDNA3.1 expression vectors, encoding the extracellular domain of the FcγR combined with a c-terminal 10xHis-tag and Avi-tag, were designed and expressed in human embryonic kidney (HEK) 293F cells. The supernatant was harvested five days post transfection and the FcγRs were isolated and purified using the ÄKTA prime plus (GE Life Sciences) equipped with His-trap column (GE Life Sciences). Biotinylation was performed with 1 µM FcγRIIIa and 3.3 nM BirA ligase, overnight at room temperature.

FcγRIIIa-V158-tetramers were made by incubating 1.74 µg/mL c-terminally biotinylated FcγRIIIa-V158 monomers with 0.6 µg/mL Streptavidin-APC (Biolegend) in 1% PBS/BSA for 30 minutes at room temperature.

Flowcytometric Assay

RBCs from a single donation from one D-positive donor (donor 18-1000, phenotype [C-, c+, D+, E+, e-; R2R2] were in aliquots cryopreserved at -80 C, and used after thawing. The cells were counted using the ADVIA 2120 hematology analyzer (Siemens Medical Solutions Diagnostics), centrifuged for 2 min. at 650g and diluted to 1.5x106 x105 cells/mL in 1% PBS/BSA. 3.0x105 cells were used per well (96-wells V-bottom plate) and washed with PBS/BSA and incubated with 50 µL MoAb- or serum dilutions, in duplo for 30 minutes at 37°C. Serum samples were used in 25% dilution in modified low ionic strength solution, ID-Diluent 2 (Bio-Rad Laboratories). The glycoengineered anti-D monoclonal antibodies were used in a 1/5 dilution series, between 5-0.0016 µg/mL in ID-Diluent 2. Mixtures of fucyosylated and afucosylated MoAbs were used to simulate different fucosylation levels. After incubation, the cells were washed 4x with PBS/BSA, and split into two populations (1.5x105 cells) for different secondaries. The cells were incubated with 50 µL of either a mixture of DyLight™ 405 AffiniPure Fab Fragment Goat Anti-Human IgG (1/400 in PBS, Jackson ImmunoResearch Laboratories) + CellTrace™ CFSE Cell Proliferation Dye (1/2000 in PBS, Invitrogen™, ThermoFisher Scientific) or FcγRIIIa-tetramers (1/3 dilution in PBS/BSA) for 30 minutes at room temperature. The cells were incubated separately with the different secondaries due to overlapping binding epitopes. To reduce flow cytometry measuring time, the cells incubated with the anti-IgG + CellTraceTM mixture and the FcγRIIIa-tetramers were combined and measured simultaneously. The anti-IgG subclass specific measurements were performed in independent experiments. The flow cytometry data were analyzed using FlowJo v10.8.1 (BD Biosciences). The RBCs were gated based on the FSC-A/SSC-A and the single cells were selected (FSC-H/FSC-

A). Then, two different populations were selected based the CellTraceTM fluorescent intensity. The geometric-mean fluorescence intensities (gMFI) of all parameters were calculated, the gMFI of the anti-IgG Dylight405 was calculated on the CellTrace™-positive population and the gMFI of the FcγRIIIa-tetramers on the CellTrace™-negative population. To facilitate the analysis of data across multiple experiments, normalization was employed to account for inter-experimental variability introduced by i.e. different flow cytometers. This approach ensured comparability, and minimized experimental biases. Normalization was performed for each flow cytometric parameter using the gMFI at the highest concentration of afucosylated MoAb IgG1 F0 (5 µg/mL), which was set to 100% for each experiment.

IAT and Monocyte-ADCC

Indirect antiglobulin titers (IAT) were determined according to standard procedures of Sanquin Diagnostic Services with a panel of D-positive donor RBCs. IAT results are provided as the maximum dilution for which agglutination could still be detected in tubes using R2R2 RBCs.

The monocyte-ADCC assay was performed following standard procedure of Sanquin Diagnostic Services, as described previously.[17,29] In short, D-positive donor RBCs were labeled with chromium-51 (51Cr), washed and incubated with undiluted AB serum or serum containing RBC alloantibodies for 60-90 minutes at 37°C. The opsonized cells were extensively washed, centrifuged and incubated with pooled monocytes, originating from 70-100 donors, for 90 minutes at 37°C and 5% CO_2. The samples are centrifuged and the 51Cr-activity was measured in the supernatant and expressed as percentage of lysis, calculated from a calibration curve based on the amount of 51Cr-activity when incubated with different dilutions of pooled polyclonal anti-D standard.

The maximum maternal antibody titer and ADCC test results were used to assess predictive values.

Statistics

Statistical analyses were performed using GraphPad Prism 9.3.1. Continuous data was presented as median [interquartile range] and categorical data as proportions. Curve fitting was performed using nonlinear regression dose-response curves with log(agonist) vs. response – variable slope (three parameters). Depending on the number of parameters, the differences in median were tested using the nonparametric Mann-Whitney test or the Kruskal-Wallis One-Way ANOVA. Correlations were assessed using

univariate or multivariate linear regression. Receiver operating characteristic (ROC) curves were made with the Wilson/Brown method. To evaluate differences in population distribution, the Chi-square test was used. For all analyses, the level of significance was set at $p \leq 0.05$. *,**,*** and **** denote a statistical significance of $p<0.05$, ≤ 0.01, ≤ 0.001 and ≤ 0.0001, respectively.

Ethical considerations

The medical ethics committee Leiden-Den Haag-Delft approved the study (P15.101/NV/nv). Written informed consent was obtained from all mothers included in this study. The study adhered to the Declaration of Helsinki and complied with the General Data Protection Regulation.

RESULTS

Study population

All pregnancies included in the OPZI2.0 cohort were assessed for their eligibility to be included in this study. Figure 1 shows the derivation of the study population. To evaluate potential selection bias, we retrieved serological data from cases that were excluded due to an absence of informed consent and from cases excluded due to insufficient remaining material. These characteristics are reported in Supplemental Table 3 and reveals that the cases included in this study generally have higher serological test results, in comparison to those excluded due to a lack of informed consent.

IgG-Fc-glycosylation of anti-D and disease severity

Anti-D specific IgG was purified from sera from 84 pregnant women and was analyzed for IgG-Fc-glycosylation by mass-spectrometry. After excluding 20 eluates due to low mass-spectrometry signal intensities, we evaluated quantities of the 20 most abundant IgG1-Fc glycopeptides and 15 most abundant IgG2/3-Fc glycopeptides (Supplemental Tables 1 and 2) in the remaining set representing 64 pregnancies from 64 different women. Table 1 presents the baseline characteristics of these 64 pregnancies. In this mass-spectrometric analysis we cannot distinguish between IgG-derived glycopeptides originating from IgG2 and IgG3 in this mostly Dutch European population.[6,30,31] However, as anti-D is known to be mostly of IgG1 and/or IgG3 and almost never of IgG2 subclass, we assume these signals to be derived from IgG3. Therefore, hereinafter we refer to IgG2/3 as IgG3.

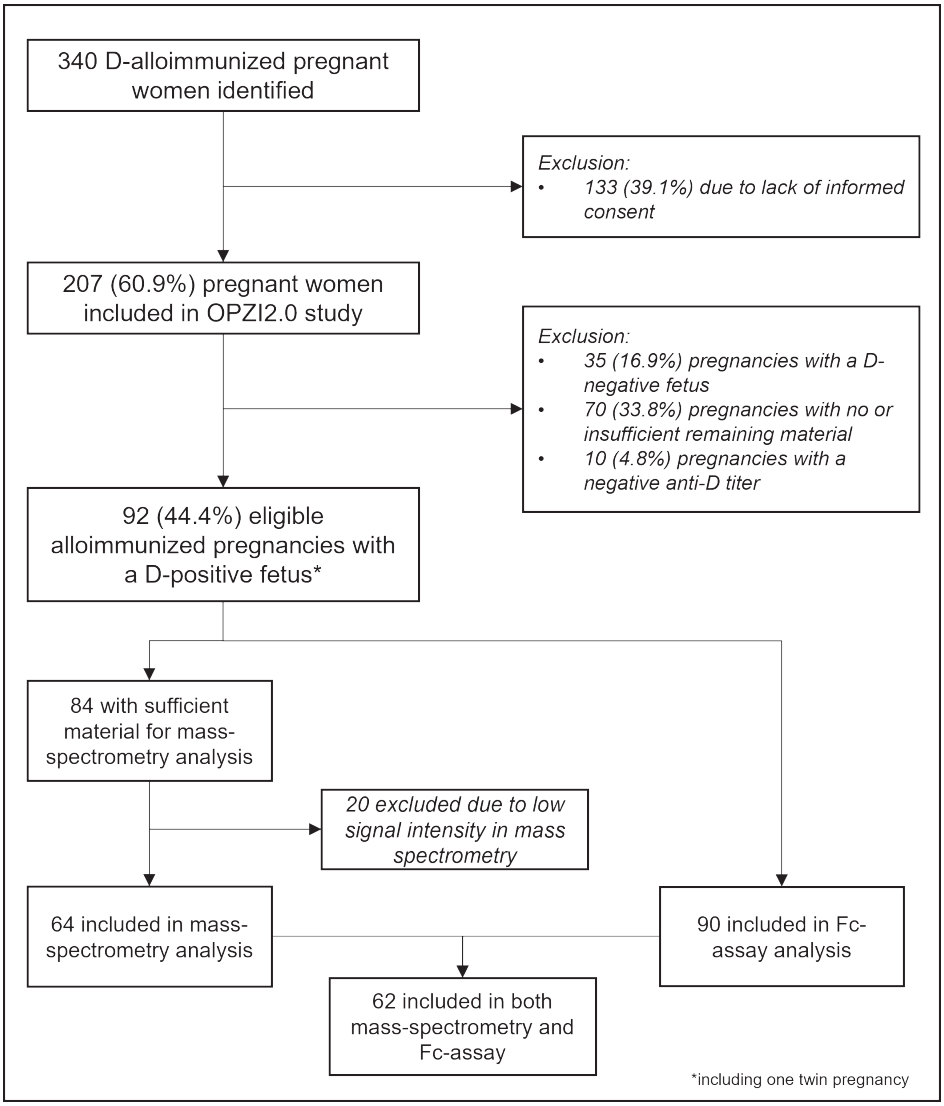

Figure 1: Derivation of the study population.

Systematic analysis of Fc-glycosylation revealed highly variable levels of fucosylation in anti-D IgG1 and anti-D IgG3 compared to total IgG1 and total IgG3, respectively (Figure 2B-C). Although the correlation was weak, there was a significant association between anti-D IgG1 fucosylation and anti-D IgG3 fucosylation (P = 0.017, R2 = 0.108) (Supplemental Figure 1). Additionally, although less pronounced, significant differences in anti-D IgG1 galactosylation, bisection and sialylation were found in comparison to total IgG1 (Supplemental Figure 3). We also identified a weak but significant correlation

between anti-D IgG1 and anti-D IgG3 sialylation (P = 0.035, R2 = 0.086), and no correlation between anti-D IgG1 and anti-D IgG3 galactosylation (P = 0.417, R2 = 0.013). Anti-D IgG3 galactosylation and sialylation was similarly significantly lower in comparison to total IgG3. We did not detect anti-D IgG3 bisection in this analysis. Analysis of IgG1 and IgG3 signal intensities revealed that, overall, IgG1 signal intensities were higher in comparison to IgG3 (Figure 2D).

As lower levels of anti-D IgG1-Fc-fucosylation have been shown to be associated with lower fetal or neonatal hemoglobin levels in HDFN[7], we investigated whether IgG1- and IgG3 anti-D fucosylation levels could distinguish between pregnancies requiring no transfusions, pregnancies with IUT and pregnancies with postnatal transfusions only (Figure 3). The area under the curve in the identification of pregnancies with IUT was 0.63 for IgG1 anti-D fucosylation and 0.54 for IgG3 fucosylation levels. Although anti-D IgG1 and IgG3-Fc-fucosylation tended to be lower in groups requiring transfusions, no significant difference in fucosylation or other glycan traits was detected.

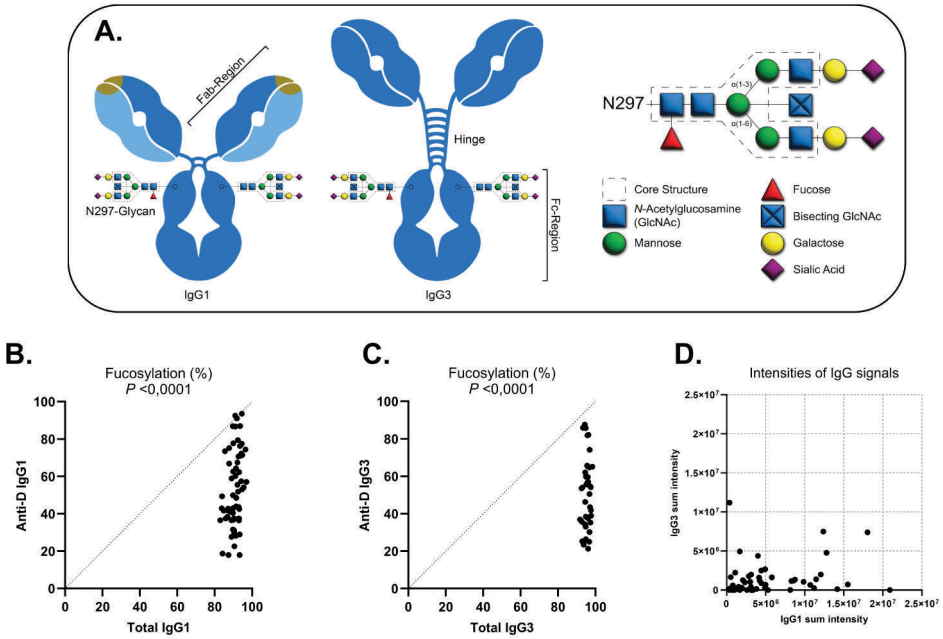

Figure 2: A) Principle of IgG-Fc-glycosylation at position 297 in the CH2-domain of the Fc-region in IgG1 and IgG3. The glycan core structure may be extended upon by fucose, bisecting GlcNAc, galactose and sialic acid. **B-C)** Anti-D IgG1 and anti-D IgG3 show highly variable and lowered Fc-fucosylation in comparison to total IgG1 and total IgG3, respectively. **D)** shows the distribution of IgG1 and IgG3 sum intensities as measured by LC-MS.

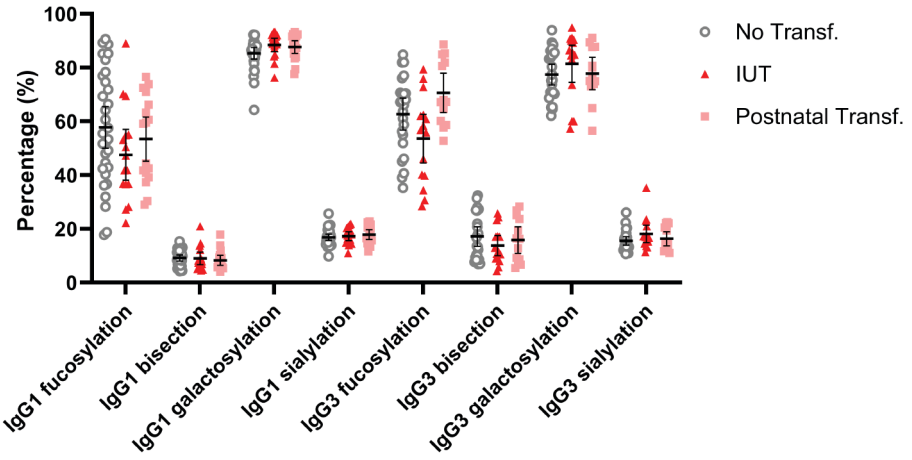

Figure 3: Fc-fucosylation, Fc-bisection, Fc-galactosylation and Fc-sialylation of anti-D IgG1 and IgG3 are not statistically significantly different between pregnancies with intrauterine transfusion and pregnancies without intrauterine transfusions. A statistically significant difference was found in Fc-fucosylation levels of anti-D IgG3 in pregnancies with intrauterine transfusions compared to pregnancies without intrauterine transfusions ($p = 0.014$).

A novel flowcytometric assay to predict disease severity

Numerous factors, such as IgG-Fc-glycosylation profiles, but also antibody quantity, antibody subclass and fetal FcγRIIIa affinity, may potentially play a pivotal role in the complexity of fetal hemolysis. We, therefore, designed a flowcytometric assay to predict disease severity by determining total anti-D IgG, IgG subclass specific opsonization levels and Fc-fucosylation dependent effector functions using FcγRIIIa-V158-tetramers (Figure 4A, Supplemental Figures 4-7). To validate the assay, we used glycoengineered anti-D moAb with either hIgG1 or hIgG3 subclass and produced those with varying fucosylation levels. As expected, these modifications did not influence binding of the moAbs to RBCs (Figure 4B-C). The FcγRIIIa-V158 binding rate indeed differed between varying levels of fucosylated antibodies and IgG subclasses, with higher FcγRIIIa-158V binding in low fucosylated hIgG1 and hIgG3 moAbs compared to highly fucosylated IgG (Figure 4D-G). Correlations between IgG-binding, FcγRIIIa-binding, titer, and ADCC are shown in Supplemental Figure 6.

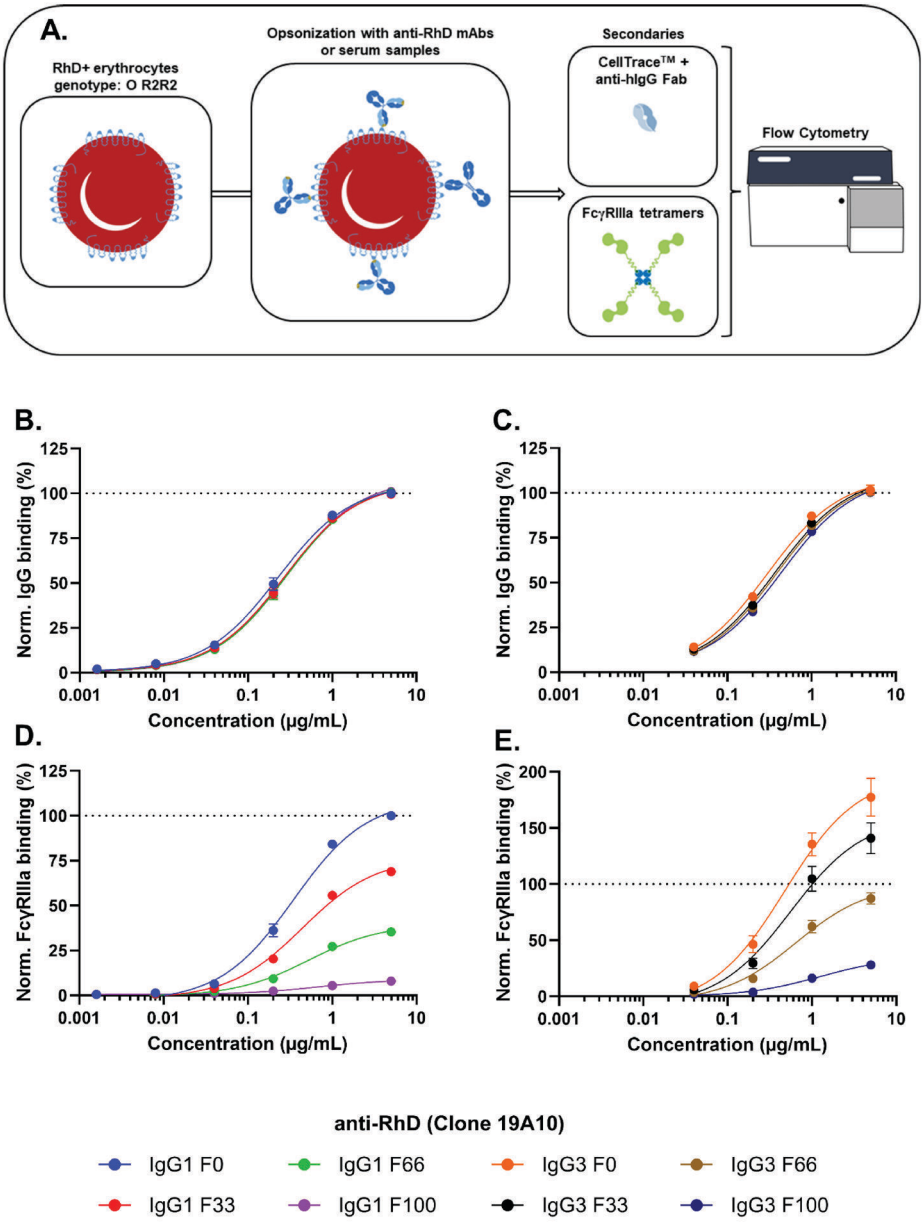

Figure 4: A) Schematic overview of a novel flowcytrometric assay to detect functionally distinct anti-D IgG using FcγRIIIa tetramers. R2R2 donor erythrocytes were used for opsonization throughout. Glycoengineered anti-D hIgG1 and hIgG3 moAbs with varying fucosylation levels were used to validate the assay. Data was normalized to the result of the highest concentration of afucosylated MoAb IgG1 F0 set at 100% (dotted lines). Anti-D hIgG1 (B) and hIgG3 (C) moAbs display similar relative IgG binding levels at varying concentrations. Binding of FcγRIIIa-tetramers to anti-D hIgG1 (D) and hIgG3 (E) moAbs, showed increasing binding to IgG with lower fucosylation.

Predictive value of IgG binding and FcγRIIIa binding

Sera from a total of 90 D-alloimmunized pregnancies were included to evaluate the predictive value of IgG- and FcγRIIIa binding. Baseline characteristics are depicted in Table 2 and did not differ compared to the pregnancies included in mass-spectrometry analysis. Multiple linear regression revealed a significant negative association between FcγRIIIa binding and anti-D IgG1 fucosylation ($P<0.001$), but not for FcγRIIIa binding and anti-D IgG3 fucosylation ($P=0.111$).

We evaluated the predictive value of the standard-of-care maximum titer and monocyte ADCC values, IgG binding and FcγRIIIa binding (Figure 5) in relation to the occurrence of fetal anemia requiring IUT and the need for postnatal transfusions in neonates without IUT, compared to pregnancies requiring no transfusion. Significant differences in IgG- and FcγRIIIa binding were found between pregnancies without transfusions and with IUT, suggestive of a potential predictive value for the need for IUTs.

We used ROC curves to further assess and quantify the predictive value of IgG binding and FcγRIIIa binding to identify pregnancies requiring IUT, in comparison to the standard of care titer and monocyte ADCC (Figure 6A). At 100% sensitivity, thereby identifying all 17 pregnancies requiring IUT among all 90 included pregnancies, the false-positivity rate was 74.2% for the maximum titer at a cut-off of 1:16 and 79.4% for the maximum ADCC at a cut-off of 50%. In the Netherlands, however, the ADCC and titer are simultaneously used to timely detect at-risk pregnancies defined as pregnancies with a titer of 1:16 or above or an ADCC reaching or exceeding 50%. Using these predetermined cut-off values, we reached 100% sensitivity for the identification of pregnancies requiring IUT with a false-positive rate of 74.6%. In comparison, the false-positivity rate of IgG binding was lower. At 100% sensitivity, thereby identifying all pregnancies requiring IUT, the false-positivity rate for IgG binding was 20.8% at a cut-off value of 22% for IgG binding, and 43.1% for FcγRIIIa binding at a cut-off value of 7% (Supplemental Table 4).

Similarly, ROC curves were used to assess the predictive value to identify neonates requiring only postnatal transfusions among all included pregnancies (Figure 6B). The curves for titer (AOC = 0.81), ADCC (AOC = 0.72), IgG-binding (AOC = 0.73) and FcγRIIIa-binding (AOC = 0.71) appeared to be comparable.

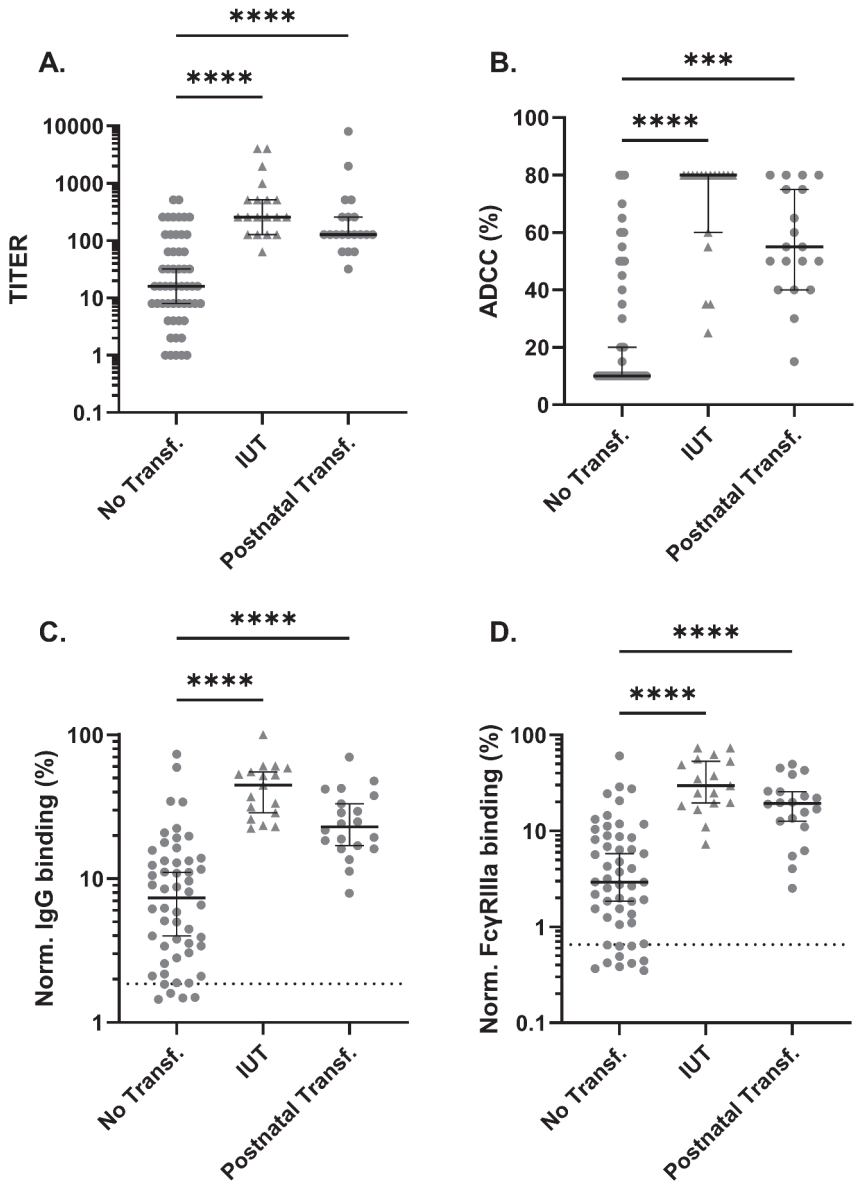

Figure 5: Visual overview of the distribution of the titer (A), ADCC (B), total IgG opsonization levels (C) and FcγRIIIa binding (D), all reflecting significant differences ($p \leq 0.0001$) between pregnancies treated with IUT and without transfusions thereby suggesting a potential predictive value ($p \leq 0.0001$ as determined in a Kruskall-Wallis test. Dotted line represents negative controls plus three standard deviations.

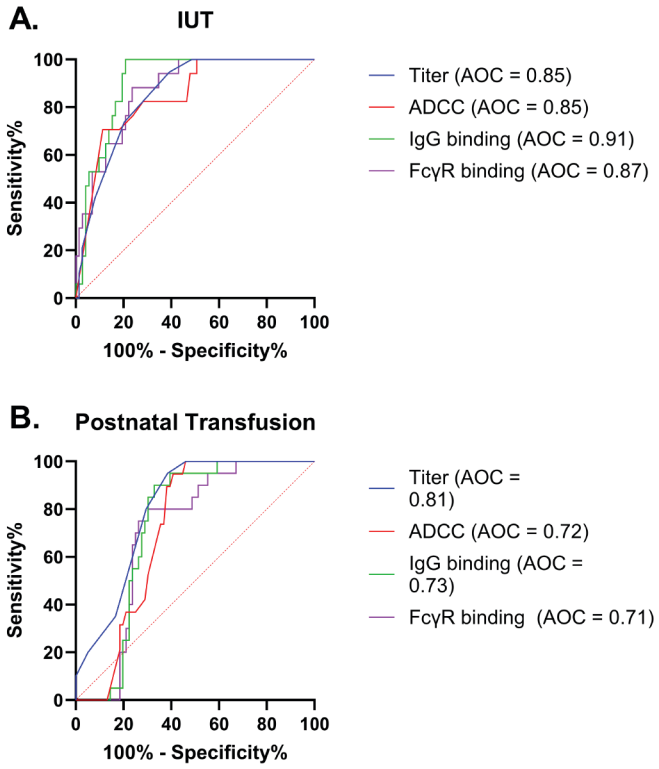

Figure 6: Anti-D IgG binding levels display a relatively high sensitivity and specificity in the prediction of intrauterine transfusions (A), and similar sensitivity rates for exchange transfusions or simple transfusions in neonates not requiring fetal therapy (B) in comparison to standard-of-care titer and ADCC. FcγRIIIa binding displays similar sensitivity and specificity rates in all fields compared to standard-of-care titer and ADCC. AOC = Area under the curve.

DISCUSSION

In this study we aimed to evaluate the association of IgG-Fc-fucosylation to the disease severity in D-mediated HDFN and we aimed to compare the clinical value of the flowcytometric assay for the prediction of severe HDFN to that of the standard maternal antibody titer and monocyte-ADCC-assay. A limitation of the study is an overrepresentation of high-risk cases in the cohort and the technological drawback to isolate sufficient anti-D from low-titer sera. Nevertheless, Fc-fucosylation in this cohort tended to be lower in pregnancies with IUT, in line with our previous observations.[7,12,31] But, in this new cohort no distinct difference in levels of afucosylated IgG that could be employed in clinical practice to accurately identify at-risk pregnancies was found, potentially owing to a certain level of selection bias. Considering this, we subsequently successfully developed a novel flowcytometric assay to accurately identify pregnancies at-risk of fetal anemia. We found that fetal anemia did not occur in D-alloimmunized pregnancies if test results showed

an IgG binding below 22%, whilst also considerably limiting the number of false-positive results in comparison to standard of care. However, it should be noted that we tested only a single timepoint in the flow-cytometric assay, whereas we applied the maximally reached value for the titer and ADCC, which might have resulted in an overestimation of cases at risk. Furthermore, the manual tube assay used to quantify anti-D titer might be less accurate and show intra-assay variation, which is solved by the more objective flowcytometric assay in which the sera are diluted in LISS buffer to increase binding. These findings can facilitate further optimization of serial serological monitoring in HDFN.

Previous findings in a small cohort showed a weak but significant correlation between low anti-D IgG-Fc-fucosylation and lower fetal or neonatal hemoglobin levels.[7] We hypothesized that pregnancies at risk of fetal anemia could be identified with anti-D IgG-Fc-fucosylation and we sought to quantify this association and potential clinical value in a large prospectively collected cohort. Similarly to Kapur et al., we found highly variable and lower anti-D IgG1-Fc-fucosylation compared to total IgG1. Additionally, we found that anti-D IgG3 also shows similar low Fc-fucosylation compared to total IgG3. Previous studies have shown that the absence of fucose on the IgG1 Asn-297 N-linked glycan enhances its affinity to FcγRIIIa dramatically with a consequent increase in ADCC.[5,10,32-36] However, we found no distinct difference between pregnancies with IUT and those without, limiting its translatability to clinical use. Also, although anti-D IgG1 and IgG3 galactosylation and sialylation - glycosylation traits known to enhance FcγRIIIa/b affinity to a relatively lower extent that fucosylation[28] – tended to be lower compared to total IgG1 and IgG3, no distinct difference between pregnancies could be detected. These results underline the fact that hemolysis in HDFN is a multifactorial process.

Serial serological monitoring in HDFN is used for timely identifying all pregnancies at risk of fetal anemia warranting sonographic monitoring, with a limited number of false-positive results and ideally also a negative predictive value to predict a low risk of expected HDFN. False-negative test results have catastrophic effects ranging from delayed treatment to fetal death. Although false-positive test results may have less disastrous consequences, it may cause unnecessary sonographic monitoring and potentially adverse psychosocial effects. Currently used predetermined cut-off values of the titer and ADCC showed, despite identifying all pregnancies with IUT, a high-false positive rate of 74.6% in the current study population. However, this may be an overestimation when compared to a previous study that found a false-positive rate of 38.7% using these thresholds.[17] This result is in part due to three IUT-treated pregnancies in the cohort, referred early in pregnancy on basis of a titer above referral thresholds, but ADCC test results still below the cut-off, without repetition of the ADCC, since it would not alter clinical management.

Even though the cohort included in this study was prospectively collected, we relied on informed consent and the availability of remaining material for analysis, which is also reflected in the number of cases excluded. A limitation of the study is that the lower proportion of included pregnancies with low titers due to a higher number of lack of informed consent in this group, which turned out to be difficult to collect from the 1st line of obstetric care providers. Nevertheless, IgG-binding and FcγRIIIa-V158-binding as determined in the novel flowcytometric assay shows a promising potential to improve the specificity of serological monitoring aiming both to identify pregnancies at risk of severe HDFN, whilst maintaining a 100% sensitivity. Theoretically, additional inclusion of cases with low titers and ADCC, and most likely no IUT (i.e. true-negative), would increase the specificity of both standard-of-care and the flowcytometric-assay. However, confirmation of these findings in an overarching and prospective cohort of D-alloimmunized pregnant women with a D-positive fetus, with few exclusion criteria, and without reliance on informed consent and remaining material to minimize selection bias, is essential to implement results into practice.

Lastly, we found that IgG-binding and FcγRIIIa-V158-binding could not accurately differentiate neonates requiring postnatal transfusions only from pregnancies requiring IUT and pregnancies not requiring any transfusions.

New developments in the field of HDFN warrant improvements in the specificity of identifying pregnancies at risk of fetal anemia. FcRn-blocking agents, such as Nipocalimab (M281), prevent transplacental transport of IgG and inhibit maternal IgG recycling causing a decrease in serum IgG.[37] If demonstrated to be effective, FcRn-blocking agents may be used early in pregnancy to delay or prevent the onset of fetal anemia.[37] Timely and accurate serological identification of pregnancies requiring IUT, with high specificity at an early gestation, is essential to limit overtreatment. But, the potential future use of FcRn-blocking agents in fetal medicine may considerably limit our capability to develop new assays to predict disease severity in HDFN.

To conclude, anti-D IgG-Fc-fucosylation levels could not distinguish between pregnancies with IUT and pregnancies without IUT in the included cohort, limiting its use in clinical practice. The flowcytometric assay reported in this study indicates that the specificity of serological monitoring in D-mediated HDFN may be improved whilst still identifying all pregnancies at-risk of an IUT. Considering selection bias, we suggest that the findings from this study should be confirmed in a prospective cohort of D-alloimmunized pregnancies within a routine setting.

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SUPPLEMENTAL FILES

Supplemental Table 1: IgG glycopeptides
Supplemental Table 1 provides the IgG glycopeptides included in the final analyte list.

Glycan composition	Isotype	Alternative nomenclature	$[M+2H]^{2+}$	$[M+3H]^{3+}$
IgGI1H3N4	IgG1	G0	1244,497622	830,0008393
IgGI1H4N4	IgG1	G1	1325,524034	884,0184473
IgGI1H5N4	IgG1	G2	1406,550446	938,0360553
IgGI1H3N5	IgG1	G0N	1346,037309	897,693964
IgGI1H4N5	IgG1	G1N	1427,063721	951,711572
IgGI1H5N5	IgG1	G2N	1508,090133	1005,72918
IgGI1H3N4F1	IgG1	G0F	1317,526577	878,6868093
IgGI1H4N4F1	IgG1	G1F	1398,552989	932,7044173
IgGI1H5N4F1	IgG1	G2F	1479,579401	986,7220253
IgGI1H3N5F1	IgG1	G0FN	1419,066264	946,379934
IgGI1H4N5F1	IgG1	G1FN	1500,092676	1000,397542
IgGI1H5N5F1	IgG1	G2FN	1581,119088	1054,41515
IgGI1H4N4S1	IgG1	G1S	1471,071743	981,0502537
IgGI1H5N4S1	IgG1	G2S	1552,098155	1035,067862
IgGI1H5N4S2	IgG1	G2S2	1697,645865	1132,099668
IgGI1H5N5S1	IgG1	G2NS	1653,637842	1102,760986
IgGI1H4N4F1S1	IgG1	G1FS	1544,100698	1029,736224
IgGI1H5N4F1S1	IgG1	G2FS	1625,12711	1083,753832
IgGI1H5N4F1S2	IgG1	G2FS2	1770,67482	1180,785638
IgGI1H5N5F1S1	IgG1	G2FNS	1726,666797	1151,446956
IgGII1H4N4	IgG2/3	G1	1309,529134	873,3551807
IgGII1H5N4	IgG2/3	G2	1390,555546	927,3727887
IgGII1H4N5	IgG2/3	G1N	1411,068821	941,0483053
IgGII1H5N5	IgG2/3	G2N	1492,095233	995,0659133
IgGII1H3N4F1	IgG2/3	G0F	1301,531677	868,0235427
IgGII1H4N4F1	IgG2/3	G1F	1382,558089	922,0411507
IgGII1H5N4F1	IgG2/3	G2F	1463,584501	976,0587587
IgGII1H3N5F1	IgG2/3	G0FN	1403,071364	935,7166673
IgGII1H4N5F1	IgG2/3	G1FN	1484,097776	989,7342753
IgGII1H5N5F1	IgG2/3	G2FN	1565,124188	1043,751883
IgGII1H4N4S1	IgG2/3	G1S	1455,076843	970,386987
IgGII1H5N4S1	IgG2/3	G2S	1536,103255	1024,404595
IgGII1H4N4F1S1	IgG2/3	G1FS	1528,105798	1019,072957
IgGII1H5N4F1S1	IgG2/3	G2FS	1609,13221	1073,090565
IgGII1H5N5F1S1	IgG2/3	G2FNS	1710,671897	1140,78369

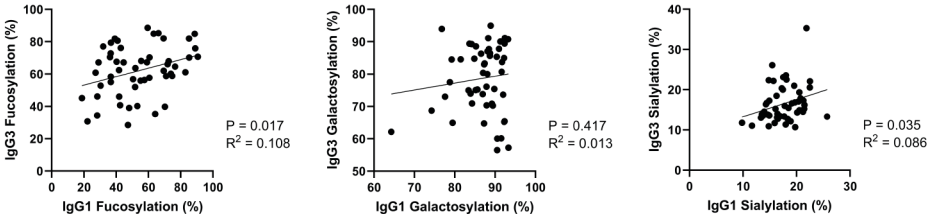
Supplemental Table 2: Calculation of IgG1 and IgG3 glycosylation traits
Supplemental Table 2 provides the description and calculation of IgG1 glycosylation traits. H: hexose, N: N-acetylhexosamine, F: fucose, S: N-acetylneuraminic (sialic) acid.

	Description	Formula
IgG1 bisection	*N*-glycans carrying a bisected *N*-acetylglucosamine	(H5N5F1S1 + H4N5F1 + H5N5F1) / sum of all IgG1 glycopeptides
IgG1 galactosylation	*N*-glycans carrying galactose(s)	(1/2 * (H4N4 + H4N4F1 + H4N5F1 + H4N4F1S1) + 2/2 * (H5N4 + H5N4F1 + H5N4S1 + H5N5F1S1 + H5N4F1S2 + H5N5F1 + H5N4F1S1)) / sum of all IgG1 glycopeptides
IgG1 sialylation	*N*-glycans carrying *N*-acetylneuraminic (sialic) acid(s)	(1/2 * (H5N4S1 + H5N5F1S1 + H4N4F1S1) + 2/2 * H5N4F1S2) / sum of all IgG1 glycopeptides
IgG1 fucosylation	*N*-glycans carrying a core fucose	(H3N4F1 + H4N4F1 + H5N4F1 + H5N5F1S1 + H5N4F1S2 + H4N5F1 + H4N4F1S1 + H5N5F1S1 + H5N4F1S1) / sum of all IgG1 glycopeptides
IgG3 bisection	*N*-glycans carrying a bisected *N*-acetylglucosamine	(H4N5 + H5N5 + H3N5F1 + H4N5F1 + H5N5F1 + H5N5F1S1) / sum of all IgG3 glycopeptides
IgG3 galactosylation	*N*-glycans carrying galactose(s)	(1/2 * (H4N4 + H4N5 + H4N4F1 + H4N5F1 + H4N4S1 + H4N4F1S1) + 2/2 * (H5N4 + H5N5 + H5N4F1 + H5N5F1 + H5N4S1 + H5N5F1S1)) / sum of all IgG3 glycopeptides
IgG3 sialylation	*N*-glycans carrying *N*-acetylneuraminic (sialic) acid(s)	1/2 * (H4N4S1 + H5N4S1 + H4N4F1S1 + H5N4F1S1 + H5N5F1S1) / sum of all IgG3 glycopeptides
IgG3 fucosylation	*N*-glycans carrying a core fucose	(H3N3F1 + H4N4F1 + H5N4F1 + H3N5F1 + H4N5F1 + H5N5F1 + H4N4F1S1 + H5N4F1S1 + H5N5F1S1) / sum of all IgG3 glycopeptides

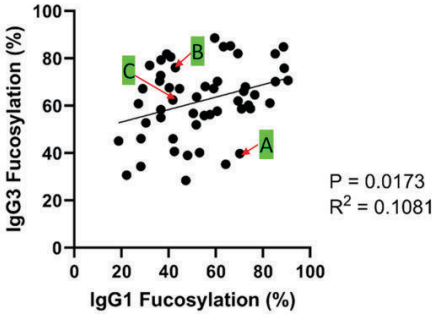
Supplemental Table 3: Characteristics of excluded cases
Supplemental Table 3 provides a detailed comparison in the available serological and clinical characteristics of cases included in the study, cases excluded due to a lack of informed consent, and cases excluded due to a lack of sufficient material.

	Included in mass spectrometry analysis (n = 64)	Included in flowcytometric assay (n = 90)	Cases excluded for no informed consent (n=133)*	Cases excluded for insufficient material (n=70)**
Serological characteristics				
Maximum Titer				
≤1:16	20.3% (13/64)	34.4% (31/90)	50.4% (57/113)	31.3% (21/67)
≥1:32	79.7% (51/64)	65.6% (59/90)	49.6% (56/113)	68.7% (46/67)
Maximum ADCC				
<50	40.6% (26/64)	53.6% (45/84)	78.8% (89/113)	56.7% (38/67)
≥50	59.4% (38/64)	46.4% (39/84)	21.2% (24/113)	43.3% (29/67)
Clinical characteristics				
Gravidity	3 [2-4]	3 [2-4]	Unknown	3 [2-4]
Parity	1 [1-2]	1 [1-2]	Unknown	1 [1-2]
Need for IUT	25.0% (16/64)	18.9% (17/90)	Unknown	20.0% (14/70)
Number of IUTs	3 [2-4]	2 [2-3.5]	Unknown	2 [2-3]
Gestational age at first IUT (weeks)	30.1 [25.4-31.4]	30.9 [27.6-32.3]	Unknown	29.3 [27.4-31.0]
Hemoglobin at first IUT (g/dL)	7.3 [5.3-9.2]	8.5 [5.6-10.8]	Unknown	8.6 [7.7-10.9]
Gestational age at birth (weeks)	37.1 [36.6-38.0]	37.9 [37.1-38.4]	Unknown	37.3 [36.6-38.4]
Birthweight (grams)	2984 [2624-3326]	3080 [2767-3453]	Unknown	3003 [2618-3490]
Hemoglobin at birth (g/dL)	13.1 [11.4-15.3]	15.2 [13.9-17.6]	Unknown	14.9 [12.3-16.9]
Female (%)	45.9% (28/61)	46.7% (14/30)	Unknown	45.7% (32/70)
Exchange transfusion	6.3% (4/64)	0% (0/31)	Unknown	11.4% (8/70)
Simple transfusion	42.2% (27/64)	0% (0/31)	Unknown	34.3% (24/70)

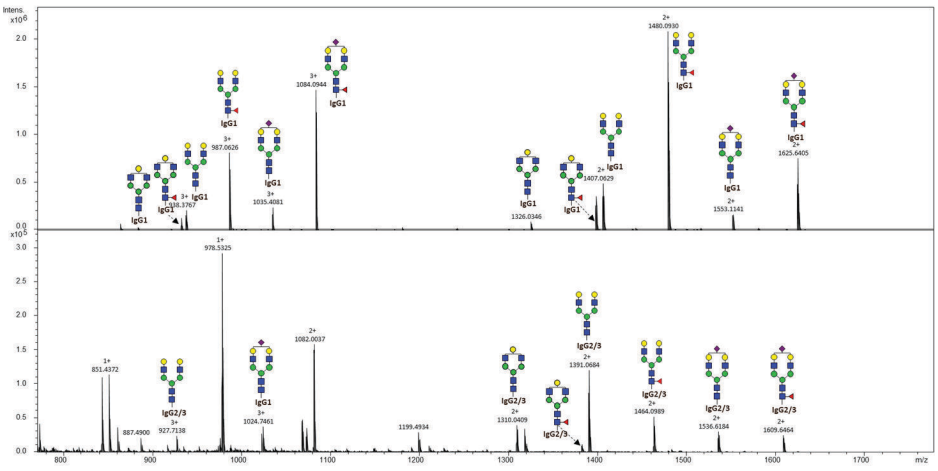
*Maximum maternal titer was missing in 2/133 cases, and the maximum maternal titer was negative (c.q. positive screen, but negative indirect antiglobulin test) in 18/133 cases.
Maximum maternal titer and ADCC unknown in 3/70 cases **Supplemental Figure 1: Correlations of anti-D IgG1 and IgG3 glycosylation traits.



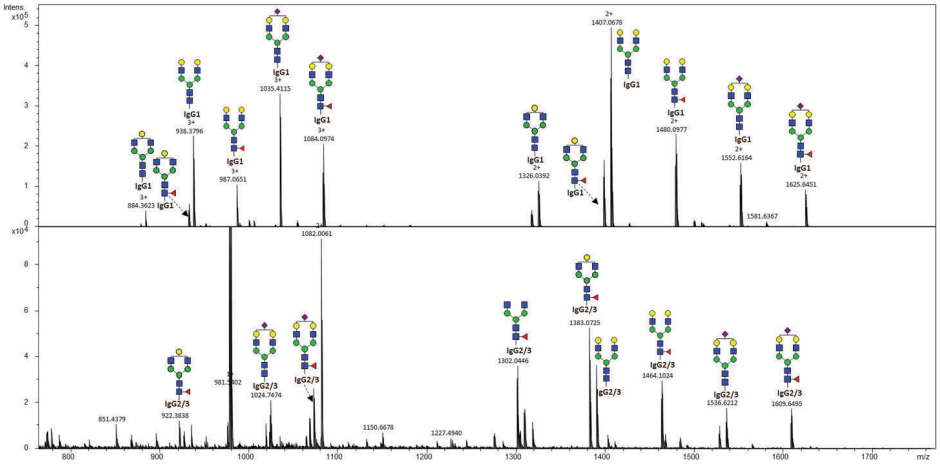
Supplemental Figure 1 shows a weak correlation between anti-D IgG1 and anti-D IgG3 fucosylation, a weak correlation between anti-D IgG1 and anti-D IgG3 sialylation, and no correlation between anti-D IgG1 and anti-D IgG3 galactosylation.



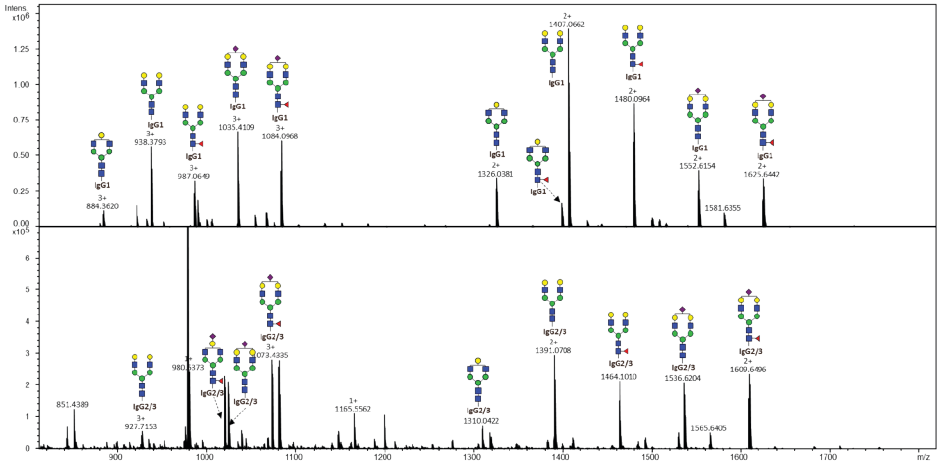
A: **Sum spectrum of Anti_D glycopeptides with high IgG1 fucosylation and Low IgG3 fucosylation**



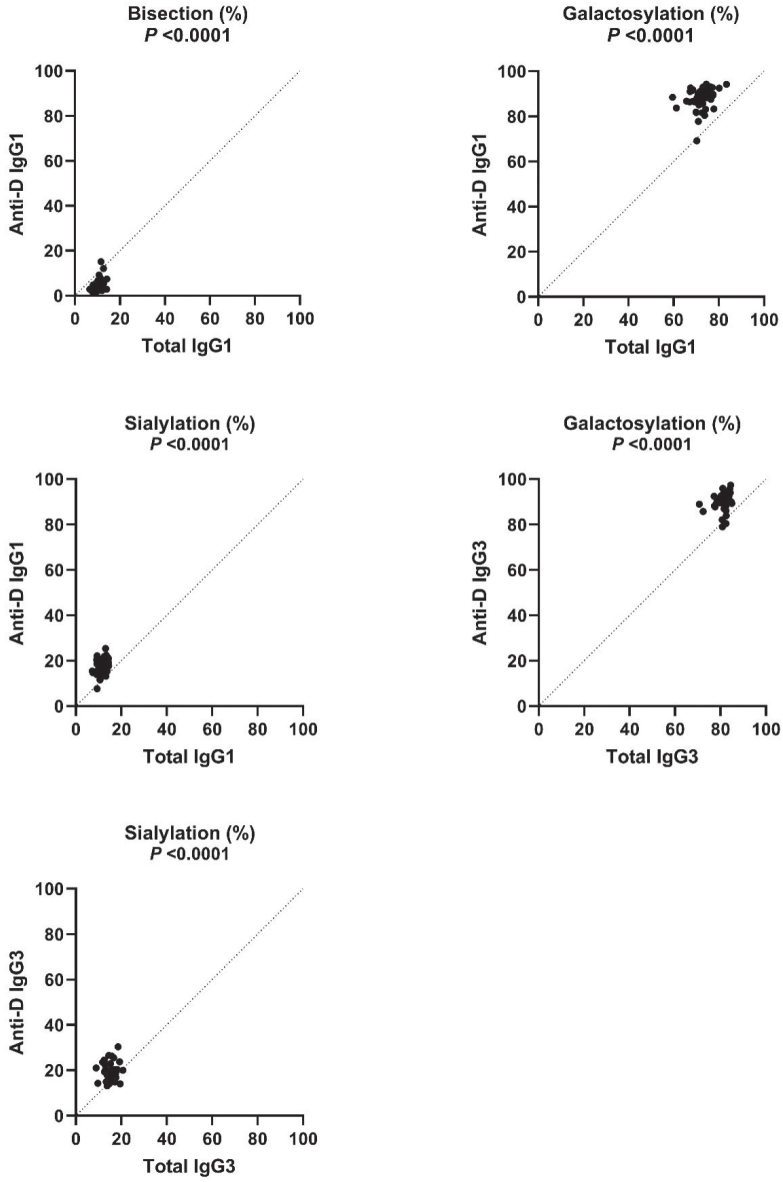
B: **Sum spectrum of Anti_D glycopeptides with Low IgG1 fucosylation and high IgG3 fucosylation**



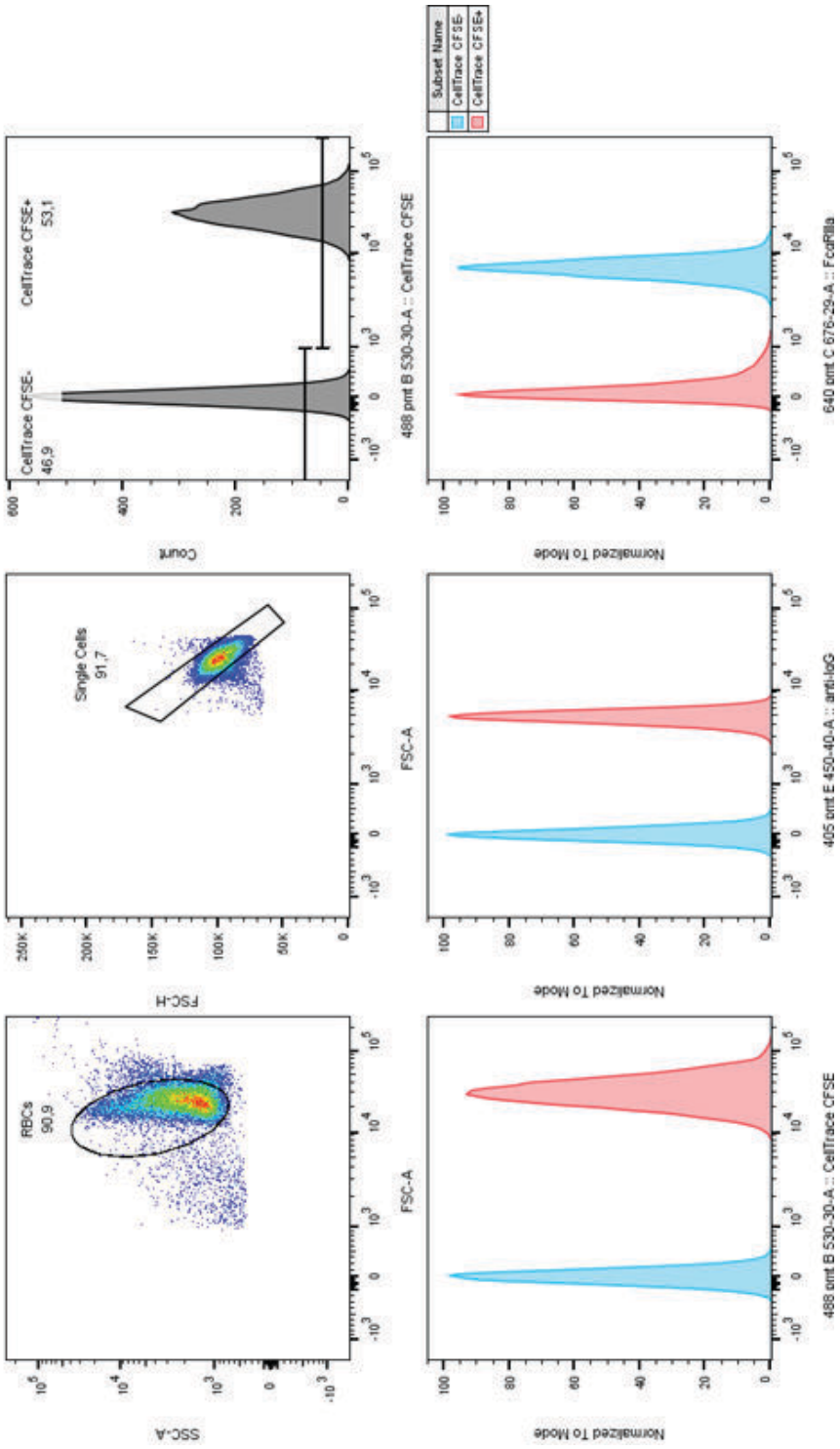
C: **Sum spectrum of Anti_D glycopeptides with Low IgG1 fucosylation and high IgG3 fucosylation**



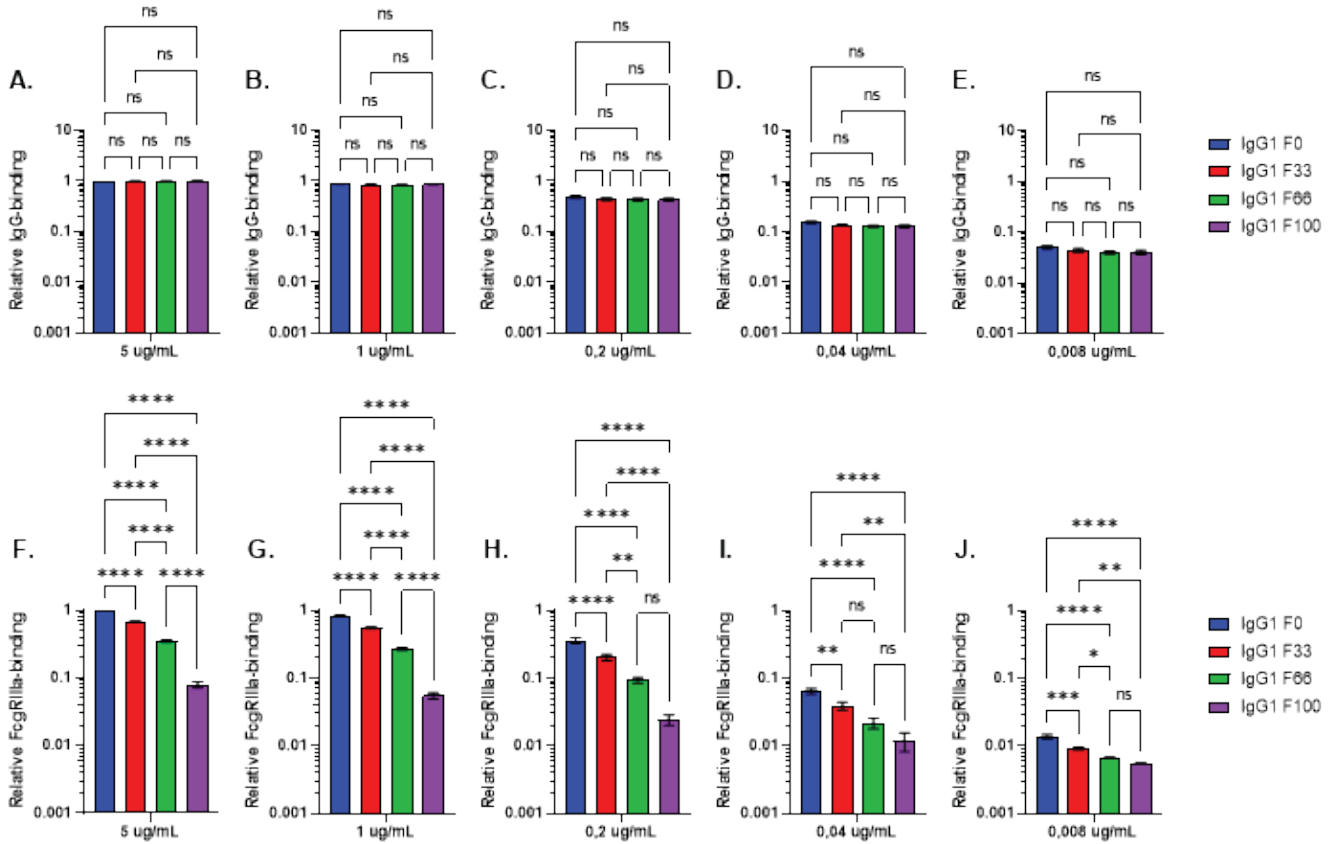
Supplemental Figure 2: Exploration of correlations between anti-D IgG1 and IgG3 fucosylation
Supplemental Figure 2 further explores the included glycopeptides from specific data points with high IgG1 fucosylation and low IgG3 fucosylation (A), low IgG1 fucosylation and high IgG3 fucosylation (B and C).



Supplemental Figure 3: Distribution of glycosylation traits
Supplemental Figure 3 shows the distribution of anti-D IgG1 and IgG2/3 glycosylation traits in comparison to total IgG1 and IgG3 glycosylation traits.

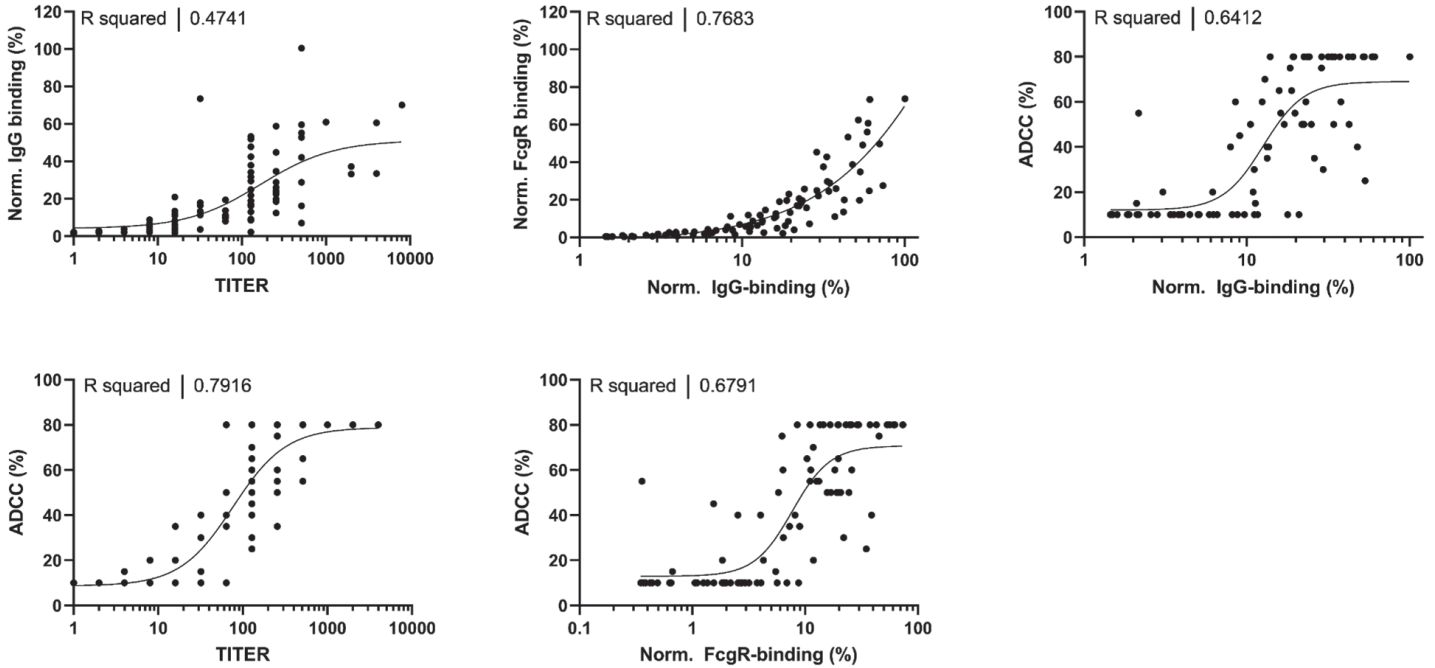


Supplemental Figure 4: Flow cytometric cell population characterization
Supplemental Figure 4: CFSE cell trace is used to characterize the cell population stained with anti-IgG.

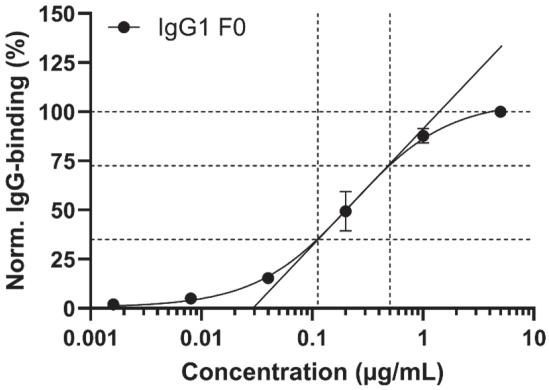


Supplemental Figure 5: IgG- and FcγRIIIa binding at varying fucosylation levels and concentrations

Supplemental Figure 5 relative IgG- and FcγRIIIa binding in varying levels of IgG1 fucosylation and concentrations. (A-E) shows stable levels of IgG-binding at varying fucosylation levels and concentrations. (F-G) shows varying levels of FcγRIIIa-binding at varying fucosylation levels and concentrations, as expected.



Supplemental Figure 6: Correlations IgG-binding, FcγR-binding, titer and ADCC
Supplemental Figure 6 shows correlations (R^2) between IgG-binding and titer, FcγR-binding and IgG binding, ADCC and IgG binding, ADCC and titer and ADCC and FcγR-binding.



Supplemental Figure 7: Linear area for IgG-binding
Supplemental Figure 7 shows the linear area for IgG-binding as determined in the flowcytometric assay.

Supplemental Table 4: Statistical metrics for IgG-binding and FcγR-binding.
Supplemental Table 4 shows the statistical metrics for IgG binding and FcγR-binding for test results set at a 100% sensitivity, thereby identifying all cases that required transfusions.

Intrauterine Transfusions		
Statistical Metric	**IgG binding**	**FcγR binding**
Sensitivity	100%	100%
Specificity	79.2%	56.9%
Positive predictive value	53.2%	35.4%
Negative predictive value	100%	100%
Area under the curve	0.91 (95%-CI 0.84-0.97)	0.87 (95%-CI 0.80-0.95)
Postnatal Transfusions		
Statistical Metric	**IgG binding**	**FcγR binding**
Sensitivity	100%	100%
Specificity	40.8%	32.9%
Positive predictive value	30.8%	28.2%
Negative predictive value	100%	100%
Area under the curve	0.73 (95-CI% 0.64-0.83)	0.71 (95%-CI 0.60-0.81)