LETTER TO THE EDITOR

# Altered glycosylation profile of anti-HPA-1a-specific antibodies: insights from a prospective fetal and neonatal alloimmune thrombocytopenia cohort

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a rare but potentially severe disorder characterized by thrombocytopenia in the fetus or neonate.[1] This is caused by maternal alloantibodies that target paternal antigens on the fetal platelets, most commonly human platelet antigen (HPA)-1a, leading to their destruction. The most severe complication is intracranial hemorrhage (ICH) which is a leading cause of morbidity and mortality in affected fetuses and neonates.[2] Higher antibody levels generally indicate more severe thrombocytopenia and risk of complications, although ICH can also occur in cases with low anti-HPA-1a levels and high antibody levels not always result in disease.[3] Nevertheless, antibody levels currently remain the only available tool to predict disease severity, highlighting the urgent need for additional biomarkers. The HPA-1a epitope is expressed on the β3 integrin which dimerizes with αIIb forming the fibrinogen receptor, expressed on platelets, or αv, creating the vitronectin receptor, expressed on endothelial cells and syncytiotrophoblasts of the placenta. Antibodies exclusively binding to αvβ3 have been suggested to correlate with ICH occurrence,[4] although these findings could not be confirmed in our recent study.[5] Another emerging area of interest influencing disease outcome in FNAIT is the highly variable fucosylation degree of anti-HPA-1a, which reflects their ability to activate FcγRIII receptors.[6,7] Immunoglobulin (Ig)G antibodies contain a conserved N-linked glycosylation site in the CH2 domain of the Fc region, where both the presence and specific composition of the glycan critically influence IgG effector functions. Although plasma IgG is almost exclusively fucosylated (~96%), reflecting that most immune responses produce nearly fully fucosylated IgG, some antigens have a greater propensity to trigger afucosylated IgG responses. This includes anti-HPA-1a antibodies, with observed fucosylation levels ranging between ~10-95%. We have previously demonstrated in a retrospective cohort that afucosylation levels inversely correlated with the severity of FNAIT suggesting increased FcγRIII-mediated effector functions to play a role.[6,7] To investigate the relationship between the anti-HPA-1a fucosylation and disease severity more in depth, we here analyzed anti-HPA-1a glycosylation profiles from the HPA-screening in pregnancy (HIP) study.[8] Unlike previous studies consisting of retrospective severe FNAIT cases, the HIP study consists of prospective serum samples without prior history of FNAIT. This design enables unbiased analysis of the anti-HPA-1a glycosylation status. Although antibody levels may change during pregnancy, we have previously found that fucosylation levels are stable within a pregnancy.[7]

The HIP samples were obtained during an observational study conducted in pregnant women in the Netherlands using left-over material from the nationwide prenatal screening program for RhD- and Rhc-negative pregnant women, collected at 27 weeks of gestation, as described.[9] The study was approved by the Medical Ethical Committee Leiden, The Hague, Delft (P16.002; *clinicaltrials gov. Identifier: NCT04067375*). To date, approaches for isolating antibodies targeting HPA-1a rely on the use of antigen-coated plates where the αIIbβ3 integrin is immobilized on solid surfaces.[6,7] While this approach is effective, it does not fully represent the functional nature of integrins *in vivo*. We therefore used HEK-293F cells stably expressing either αIIbβ3 or αvβ3 integrin, without HLA class I expression by CRISPR/Cas9 deletion, to eliminate detection of common irrelevant HLA class I alloantibodies (*Online Supplementary Figure S1A*). This method for purification of these antibodies has been described in more detail.[5] We validated this new method using ten retrospective FNAIT samples for which anti-HPA-1a glycosylation data had previously been obtained through antibody purification using platelet-derived (PAK) plate-bound antigens.[7] The Asn297-Fc glycopeptides were analyzed for both antigen-specific and total IgG (Figure 1A, B) using a nanoLC-ESI-MS setup combined with UltiMate™ 3000 RSLCnano System coupled with an Impact quadrupole time-of-flight (qTOF) mass spectrometer equipped with a nanoBooster.[10] Compared to the previous study where plates coated with β3 integrins isolated from platelets were used for isolation, the current method resulted in consistent and similar glycosylation results, with slightly higher degrees of fucosylation for both total (*Online Supplementary Figure S1B*) and anti-HPA-1a-specific antibodies, which were not due to differences in data processing (*Online Supplementary Figure S1C, D*). Slight changes were also observed for the other glycan traits, except bisection (*Online Supplementary Figure S2*). Overall, we observed a fixed offset suggesting both methods are equally suited to detect differences in fucosylation between samples, and that no major posttranslational modification differences of these integrins between HEK-293F cells and platelets exist that affect the quality of antibody purification. Next to the isolation method, also the LC-MS setup as well as the data processing has been improved since previous measurements. High resolution qTOF mass spectrometry provides more robustness against chemical background

and interfering signals than the previously used unit mass resolution ion trap. Moreover, automated data processing allows a more controlled spectral integration and data curation. Both synergistically improve precision, robustness and accuracy and are likely related to the small differences observed in this study compared to the previous study.[7,11,12] Besides mass spectrometry, we have recently developed various platforms (ELISA, SPR and flow cytometry) using FcγRIIIa as an agent to quantify fucosylation, which is well suited for future development in routine laboratories.[13]

It has been suggested that anti-HPA-1a antibodies can exert a preference for binding to the HPA-1a epitope presented on either αIIbβ3 or αvβ3 integrin complexes.[4] We therefore examined whether antibodies eluted from HEK-293F cells expressing HPA-1a in the context of αIIb or αv exhibited different glycoprofiles for anti-HPA-1a-specific IgG. The glycosylation profiles of antibodies isolated with both cell types were virtually indistinguishable (Figure 2) supporting our recent findings that the same anti-HPA-1a antibody pool bind to both αIIbβ3 and αvβ3.[5] This also reinforces the

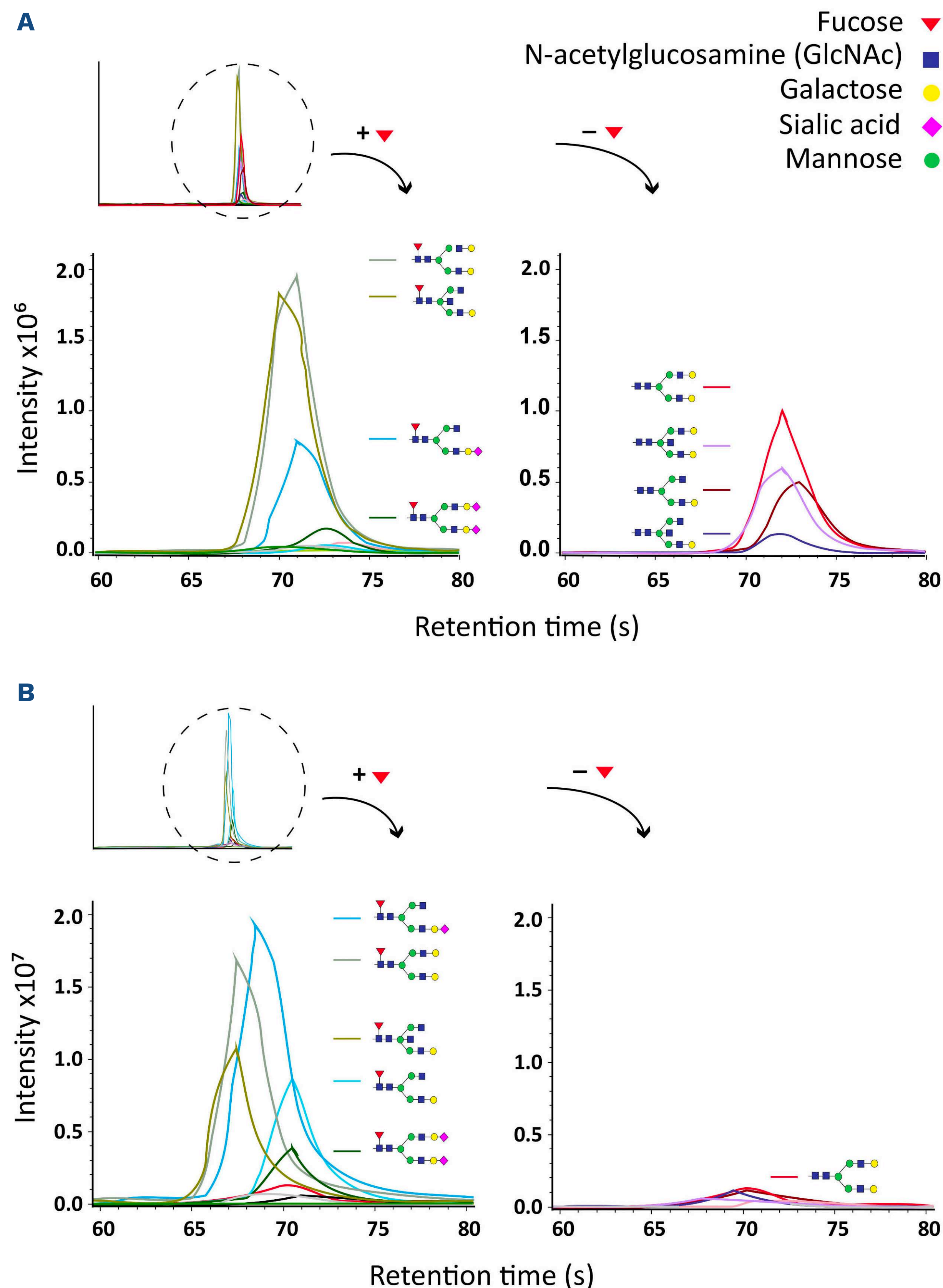
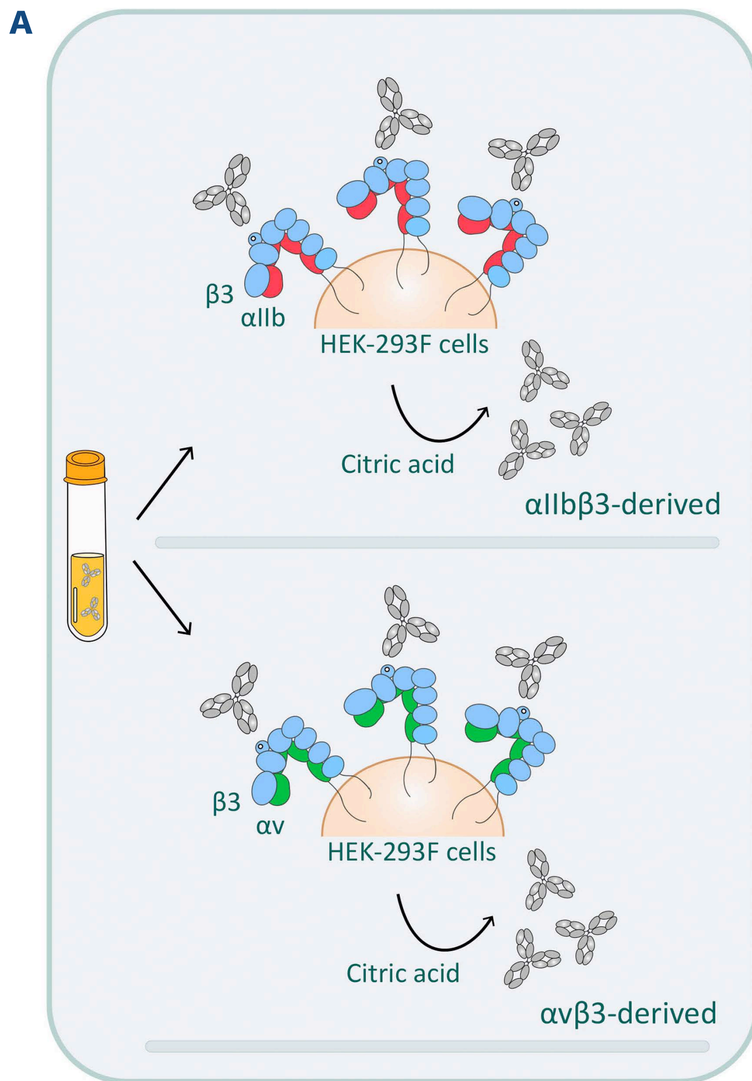
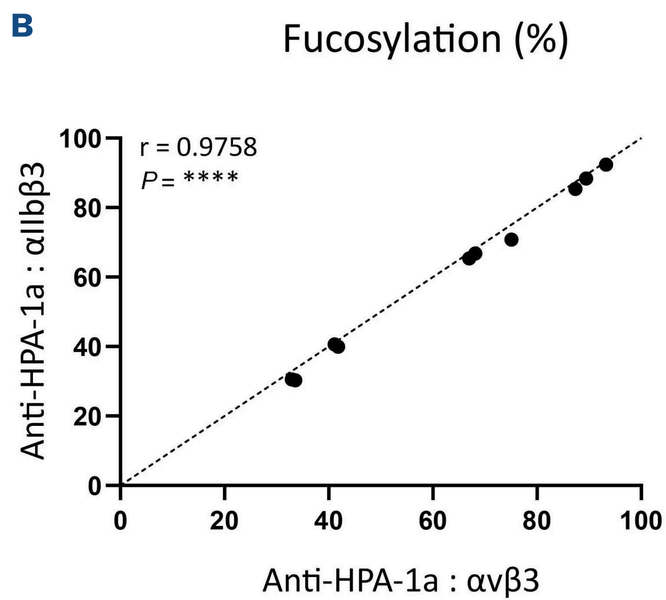
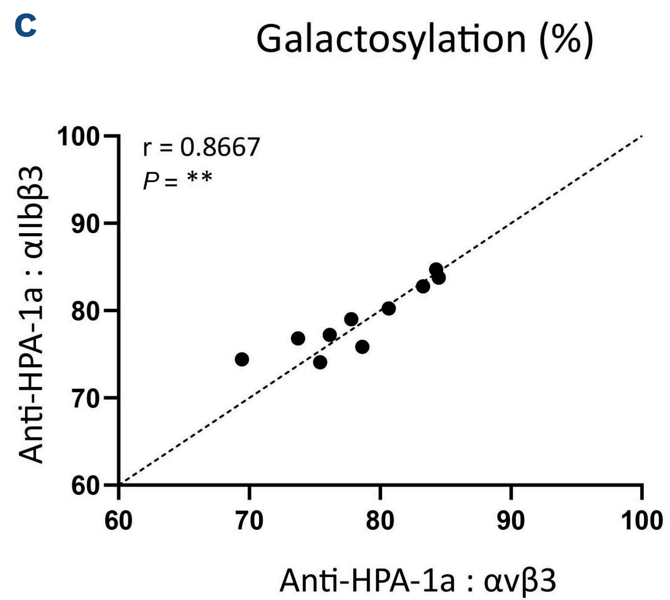
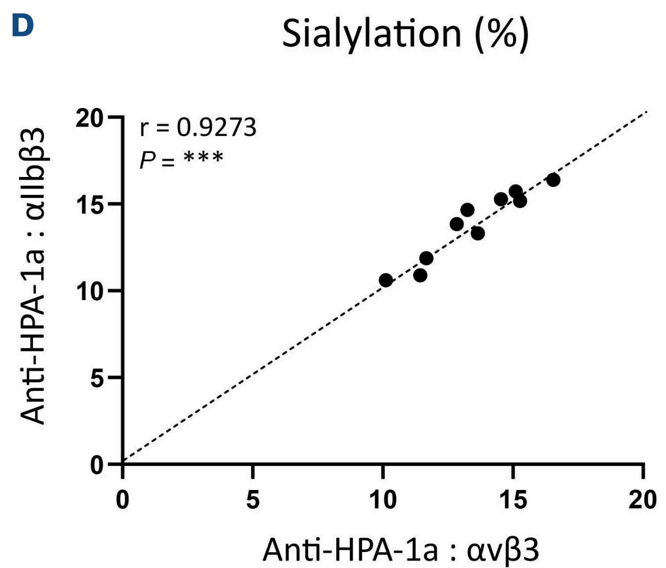



**Figure 1. Extracted ion chromatograms of IgG-Fc glycopeptides.** Chromatogram of a representative sample with lowered fucosylation is shown for human platelet antigen (HPA)-1a-specific immunoglobulin (Ig)G1, eluted from αIIbβ3-expressing HEK-293F cells, (A) and total IgG1 (B). Chromatograms are separated in fucosylated (left) and afucosylated (right) glycoforms. The most abundant glycoforms that passed the analyte curation are shown. s: seconds.



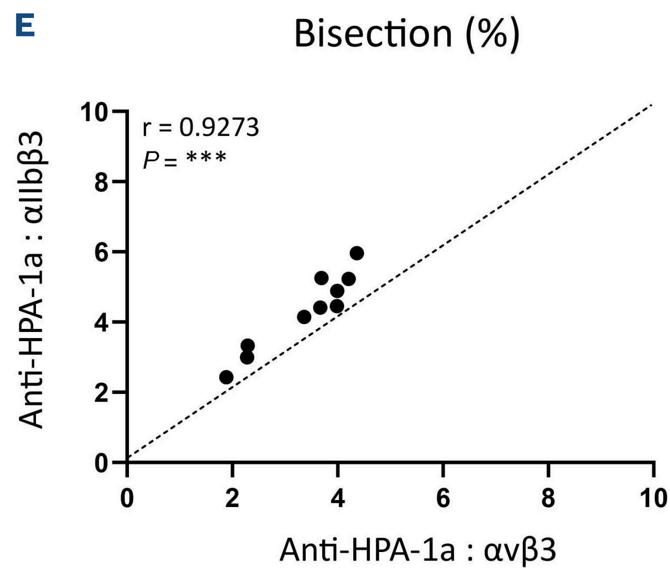




**Figure 2. Anti-HPA-1a antibodies derived from αIIbβ3- or αvβ3-expressing HEK-293F cells have comparable glycosylation profiles.** (A) HEK-293F cells stably transduced with either integrin αIIbβ3 or αvβ3, both carrying the human platelet antigen (HPA)-1a epitope, were incubated with maternal sera from Finnish fetal and neonatal alloimmune thrombocytopenia (FNAIT) cases to absorb anti-HPA-1a antibodies. After incubation, the cells were washed and specific antibodies were eluted. The immunoglobulin (Ig)G glycoprofiles were then analyzed using mass spectrometry. (B) Fucosylation, (C) galactosylation, (D) sialylation and (E) bisection were compared for antibodies derived from αIIbβ3 and αvβ3 cells. The dotted line represents the equal glycosylation degree of HPA-1a-specific antibodies derived from αIIbβ3 and αvβ3. To test correlation's, a Spearman's correlation was performed. Statistically significant differences are indicated by asterisks: ***P*<0.01; ****P*<0.001; *****P*<0.0001; NS: not significant.

robustness of the cell-based antibody purification method and the use of αIIbβ3-expressing cells for isolating anti-HPA-1a from the HIP study.

All cases (N=79) were asymptomatic except for one case which experienced severe FNAIT with intracranial hemorrhage and fetal death, and three cases with mild disease symptoms, including small bleedings on the skin, scalp or airways.[8] Of these samples, 41 samples, including the cases with clinical disease, passed the signal quality threshold for mass spectrometry analysis. The prospective nature of the HIP samples has allowed the collection of serum samples from healthy pregnancies, enabling an unbiased evaluation of the anti-HPA-1a antibody glycosylation profile. This contrasts with prior studies which relied on retrospective sampling with a history of FNAIT (Finnish cohort),[7] or with either a history of FNAIT or sporadically identified cases (Dutch cohort).[6] The anti-HPA-1a antibody levels from the HIP samples were significantly lowered in the more severely affected retrospective Finnish cohort (Figure 3A). Also, the fucosylation degree was lowered for the HIP study samples

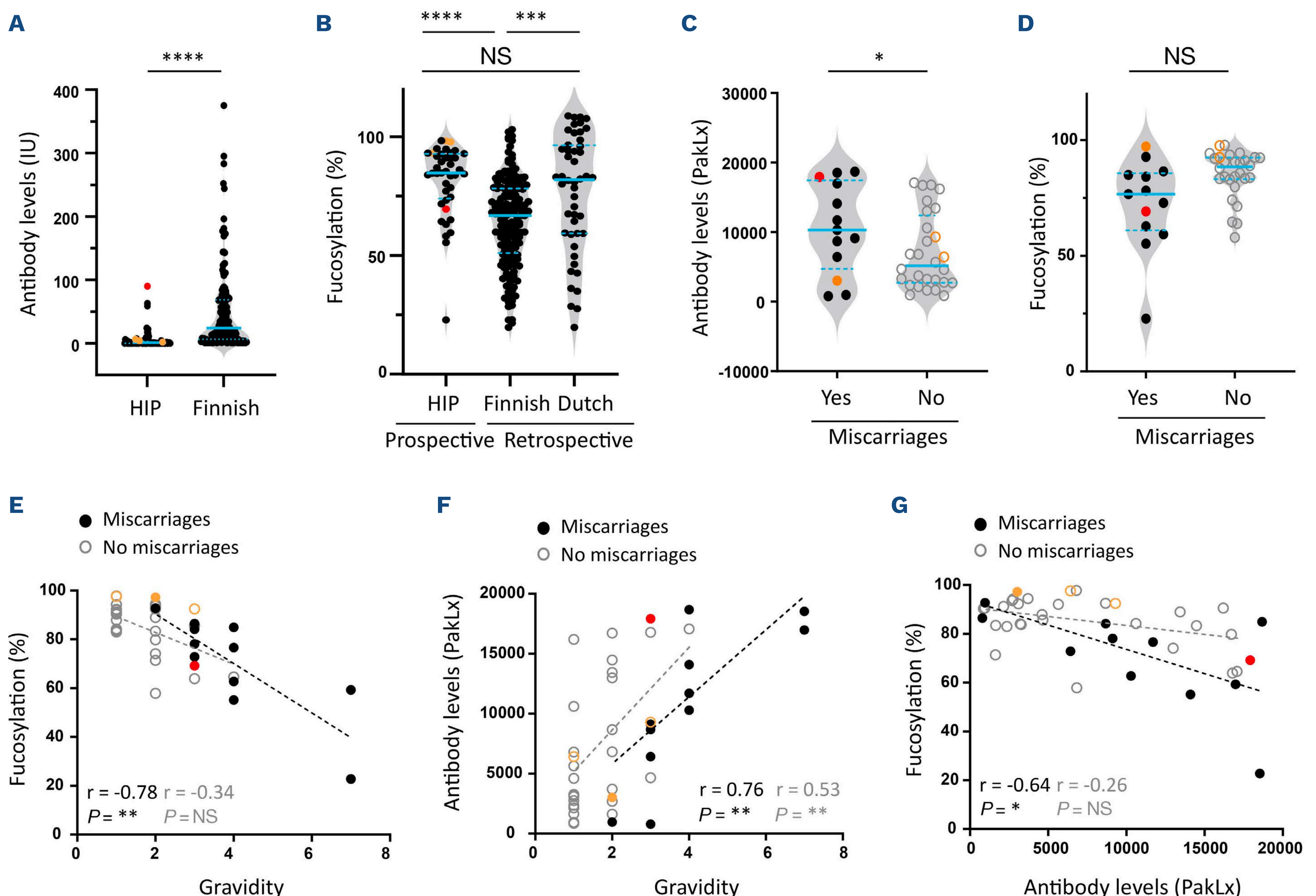



**Figure 3. Prospective fetal and neonatal alloimmune thrombocytopenia samples without disease show a higher fucosylation degree compared to affected pregnancies which is related to gravidity and antibody levels.** A total of 79 human platelet antigen (HPA)-screening in pregnancy (HIP) samples were analyzed by quantitative monoclonal antibody-specific immobilization of platelet antigens (MAIPA) and 41 samples by mass spectrometry. The antibody levels (A) and fucosylation degree (B) were compared to previous data obtained from the Finnish and/or Dutch retrospective fetal and neonatal alloimmune thrombocytopenia (FNAIT) cohorts. The fucosylation degrees of the 2 retrospective cohorts were adjusted by adding the average 10.72 deviation from 1 (*Online Supplementary Figure S1C*), to enable comparison with the new data. (C) The antibody levels and (D) fucosylation degree of the HIP samples were compared between women with and without miscarriage history. (E) A correlation was made between (E) the fucosylation degree and gravidity, (F) antibody levels and gravidity and (G) fucosylation degree and antibody levels of the HIP samples. Black/grey dots indicate cases with no clinical symptoms, orange dots represent mild cases and red dot represents severe FNAIT case, with open dots representing the no miscarriage group and closed dots the miscarriage group. The median with quartiles are depicted in blue (A-D) and significant differences were tested by Mann Whitney test (A, C, D) or non-parametric Kruskal-Wallis test (B). To test correlations in (E, G) a Spearman's correlation was performed. (E, G) The dotted lines represent simple linear regression for the miscarriages (black line) and no miscarriages (grey line) groups. The regression lines shown in (E, F) were similar while those in (G) were statistically different when comparing the intercepts. Significant differences in all panels are indicated by asterisks: *$P$<0.05; **$P$<0.01; ***$P$<0.001; ****$P$<0.0001; NS: not significant.

(*Online Supplementary Figure S3*) but less so compared to the Finnish cohort (Figure 3A, B).[7] Notably, the single severe-disease case in the HIP study had a lower fucosylation degree as well as higher antibody level compared to asymptomatic cases (Figure 3B). Only antibody levels, and not the degree of fucosylation, showed a significant difference between women with or without a history of miscarriages in the HIP cohort (Figure 3C, D). The fucosylation levels exhibited a decreasing trend with the number of pregnancies, while antibody levels showed the opposite trend, independent of miscarriage history, as indicated by statistically similar regression curves (Figure 3E, F). However, a significant correlation between afucosylation and antibody levels was observed only in pregnancies with a history of miscarriage (Figure 3G), possibly suggesting that higher responses are associated with more potent afucosylated antibodies - both traits previously associated with more severe disease.[6,7] Since severe FNAIT often presents in first pregnancies and disease severity typically does not improve in subsequent pregnancies, the observed decline in antibody fucosylation across successive pregnancies in the HIP samples is in line with these observations.

High anti-HPA-1a-specific levels have been positively associated with lower fetal platelet counts[14] which is also reflected in our study where the HIP cohort exhibits lower antibody levels compared to the retrospective cohort. However, antibody levels alone do not strictly predict disease severity or ICH occurrence.[3] Higher gravidity showed a significant correlation between both elevated antibody levels and afucosylation. This pattern contrasts with findings from the retrospective Finnish cohort, where fucosylation declined after the first pregnancy but then stabilized in subsequent ones.[7] A key difference lies in the clinical profile of the cohorts, as the Finnish cohort includes only cases with severe disease, which are linked to reduced fucosylation, whereas our cohort started off as mostly asymptomatic cases with generally higher fucosylation levels. Remarkably, a significant inverse correlation between antibody levels and fucosylation degree was observed only in individuals with a history of miscarriage. Notably, literature suggests that miscarriage is a risk factor for alloimmunization[8] as well as for severe FNAIT.[2,15] Based on this, we propose that experiencing a miscarriage may increase the likelihood of immunization which could impact the antibody response and fucosylation pattern in subsequent pregnancies.

In summary, our data supports the idea that, besides antibody quantity, qualitative characteristics, such as the fucosylation status, are also likely important determinants in predicting disease severity. For example, individuals with relatively low antibody levels but markedly afucosylated antibodies may be at higher risk than those with high antibody levels and high fucosylation. Therefore, integrating both quantitative (antibody levels) and qualitative (fucosylation) characteristics potentially offers a more accurate risk stratification framework in FNAIT.

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**Contributions**
JO, ES and GV designed the study. JO, AB, DF, WW, and JN collected experimental data. JO wrote the manuscript which was revised by all co-authors. JO, AB, DF, WW, JN, MW, SG, TV, DW, ES and GV contributed to data interpretation and approval of the final version.

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**Data-sharing statement**
We agree to share all experimental and anonymous clinical data issued from this publication.

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