haematologica

Journal of the Ferrata Storti Foundation

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

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Author contribution: JO, ES and GV designed the study. JO, AB, DF, WW, and JN collected experimental data. JO wrote the manuscript that was revised by all coauthors. JO, AB, DF, WW, JN, MW, SG, TV, DW, ES and GV contributed to data interpretation and approval of the final version.

Declaration of interest: The authors declare that they have no conflicts of interest.

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

Letter to the editor

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] IgG 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, trial registration number: 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 (Supplementary Fig. 1A). This method for purification of these antibodies has been described in more detail.[5] We validated this new method using 10 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 (Fig. 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 (Supplementary Fig. 1B) and anti-HPA-1a-specific antibodies, which were not due to differences in data processing (Supplementary Fig. 1C-D). Slight changes were also observed for the other glycan traits, except bisection (Supplementary Fig. 2). 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 FcɣRIIIa 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 (Fig. 2) supporting our recent findings that the same anti-HPA-1a antibody pool bind to both αIIbβ3 and αvβ3.[5] This also reinforces the 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 (Fig. 3A). Also, the fucosylation degree was lowered for the HIP study samples (Supplementary Fig. 3) but less so compared to the Finnish cohort (Fig. 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 (Fig 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 (Fig. 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 (Fig. 3E-F). However, a significant correlation between afucosylation and antibody levels was observed only in pregnancies with a history of

miscarriage (Fig. 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.
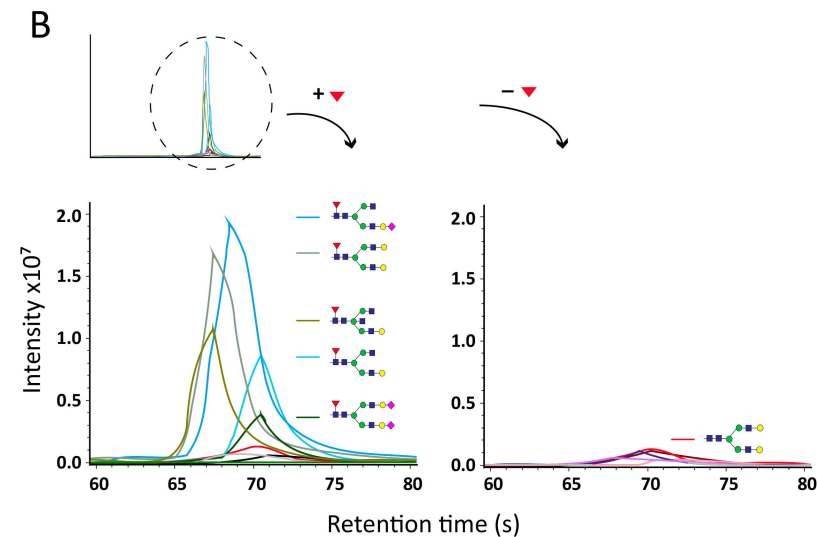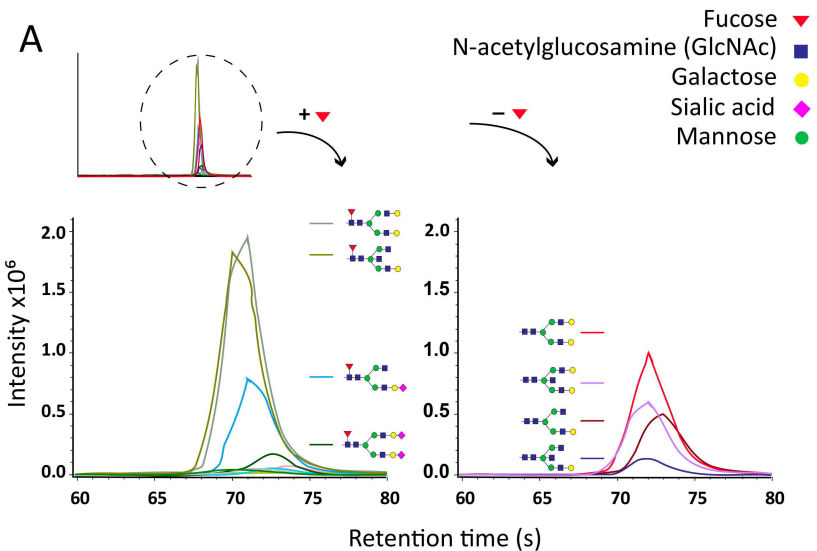
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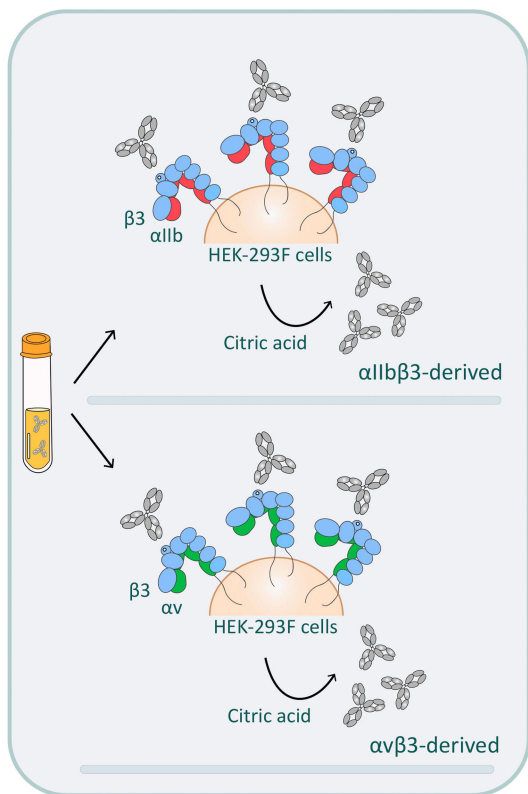
Figure legends

Figure 1: Extracted ion chromatograms of IgG-Fc glycopeptides. Chromatogram of a representative sample with lowered fucosylation is shown for HPA-1a-specific IgG1, 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.

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 HPA-1a epitope, were incubated with maternal sera from Finnish FNAIT cases to absorb anti-HPA-1a antibodies. After incubation, the cells were washed and specific antibodies were eluted. The IgG 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: **<0.01, ***<0.001, ****<0.0001, ns; not significant.

Figure 3: Prospective FNAIT samples without disease show a higher fucosylation degree compared to affected pregnancies which is related to gravidity and antibody levels. A total of 79 HIP samples were analyzed by quantitative 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 FNAIT cohorts. The fucosylation degrees of the two retrospective cohorts were adjusted by adding the average 10.72 deviation from 1 (Supplementary Fig. 1C), 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 nonparametric 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: *<0.05, **<0.01, ***<0.001, ****<0.0001, ns; not significant.
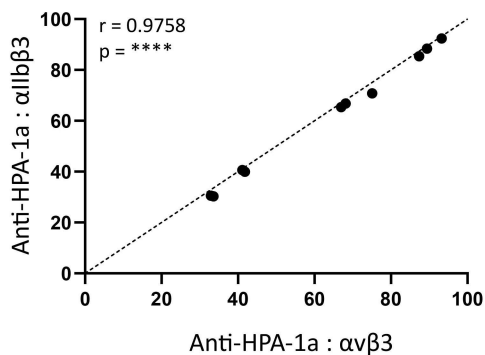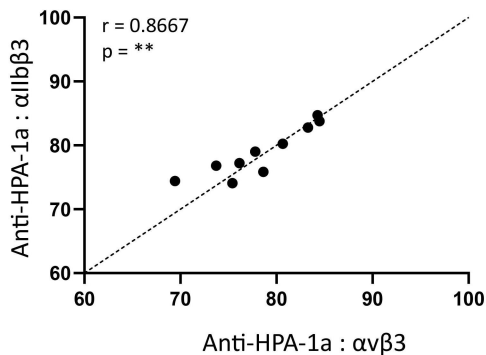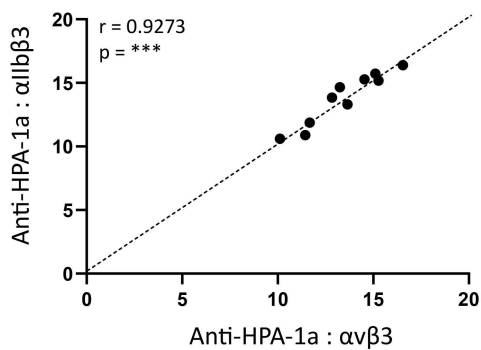
A

β3
αIIb
HEK-293F cells
Citric acid
αIIbβ3-derived

β3
αv
HEK-293F cells
Citric acid
αvβ3-derived

B   Fucosylation (%)

r = 0.9758
p = ****

Anti-HPA-1a : αIIbβ3
Anti-HPA-1a : αvβ3

C   Galactosylation (%)

r = 0.8667
p = **

Anti-HPA-1a : αIIbβ3
Anti-HPA-1a : αvβ3

D   Sialylation (%)

r = 0.9273
p = ***

Anti-HPA-1a : αIIbβ3
Anti-HPA-1a : αvβ3

E   Bisection (%)

r = 0.9273
p = ***

Anti-HPA-1a : αIIbβ3
Anti-HPA-1a : αvβ3
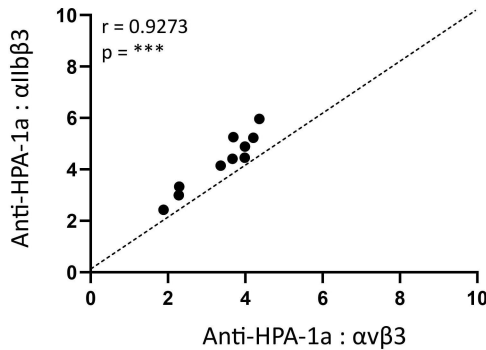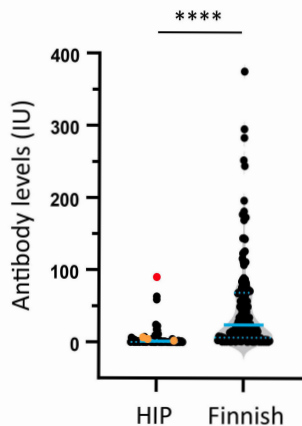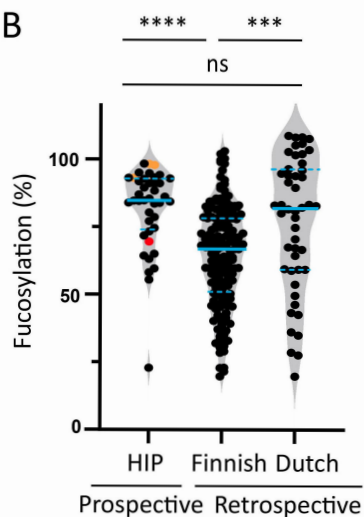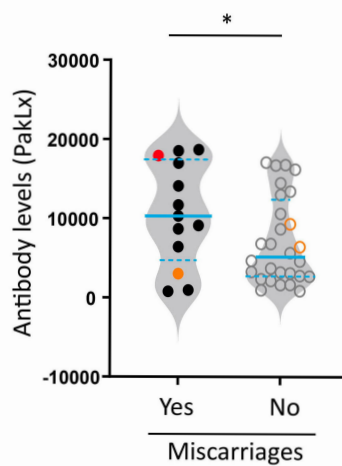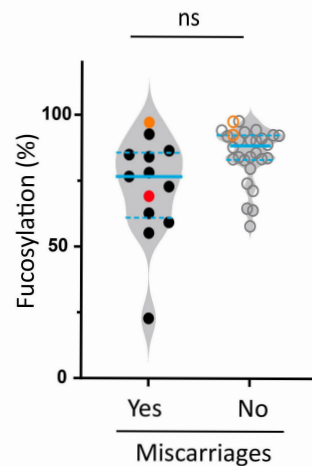
Supplementary figure legends

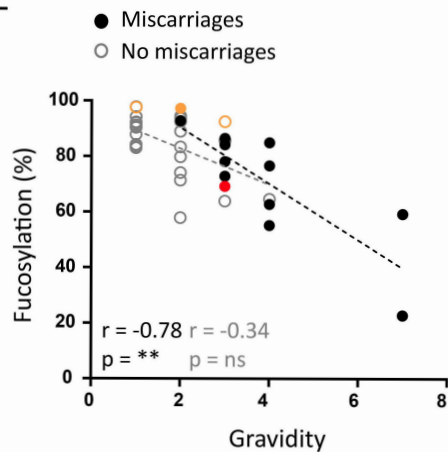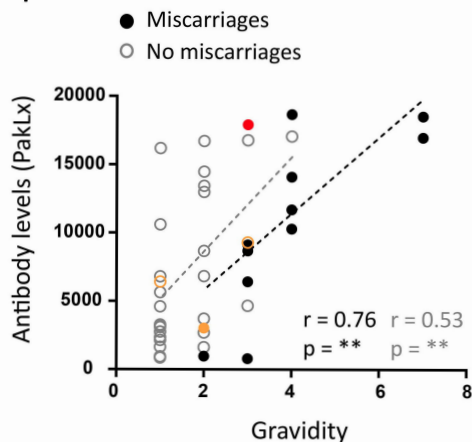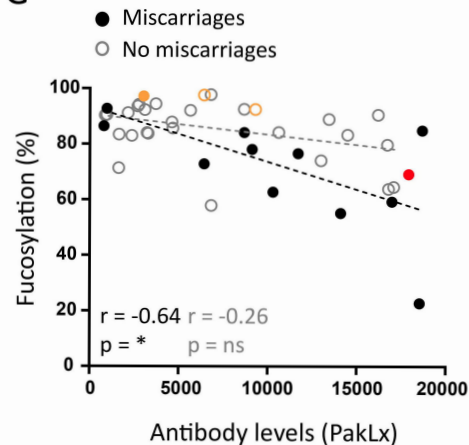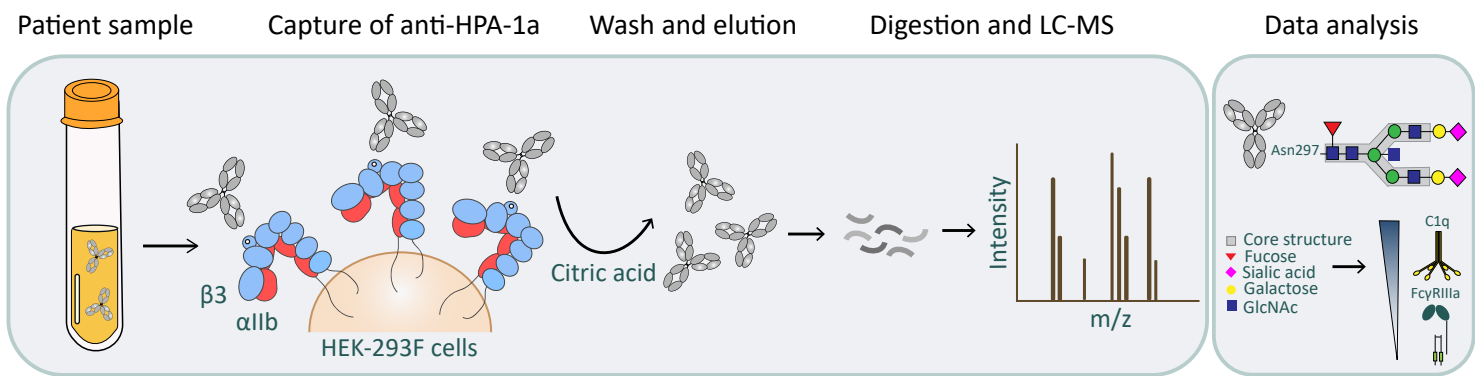**Supplementary figure 1: Isolation of anti-HPA-1a antibodies using HEK-293F cells expressing integrin αIIbβ3. (A)** Schematic overview of antibody isolation. A total of 5x105 HEK293F cells expressing either αIIbβ3 or αvβ3 were seeded and incubated with 50μl (HIP samples) or 20μl (retrospective samples) serum for 30min at 4°C to absorb all specific antibodies. Cells were washed extensively afterwards to remove aspecific proteins and unbound antibodies. Elution of bound IgG was performed with citric acid buffer (pH2.8) and subsequent neutralization was performed with Tris (pH9.0). After digestion, IgG-Fc glycans were analyzed by mass spectrometry. LC-MS signals were integrated in LaCyTools (version 2.1.0, build 20230525) and further analyzed in GlycoDash (version 1.5.3). Settings of LaCyTools and GlycoDash processing report is provided as Supplementary Information. The Fc-glycan structures of antibodies influence the binding strength to C1q, the first component of the complement system, and FcγR's, which facilitates antibody-dependent cellular cytotoxicity and phagocytosis. **(B)** Antibodies were isolated from 10 retrospective FNAIT samples from the Finnish cohort. The fucosylation degree of total and **(C)** specific IgG were compared to previously obtained data from an antigen-coated plate-based isolation method. The dotted line represents the equal fucosylation degree of HPA-1a-specific IgG between the old isolation method and current isolation method. **(D)** The current automated data processing workflow was compared to the previous largely manual data processing to observe the effect of the processing on IgG-Fc fucosylation outcome. We tested if differences in data processing were responsible for these minor alterations in glycosylation values compared with our previous work. After re-analyzing the previous original ion trap data with the new software-based workflow, no significant differences were observed. Simple linear regression was performed in (C) and slopes of the indicated equation in (C) and Y=X were compared which were not significantly different. Statistical analysis was performed using two-tailed paired t-test analysis comparing specific and total IgG glycosylation. Statistically significant differences are indicated by asterisks: ***<0.001, ****<0.0001, ns; not significant.
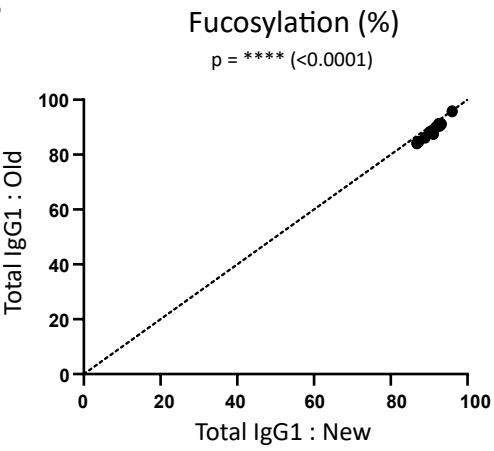
Supplementary figure 2: Glycosylation traits in the newer method are slightly higher than in the older method except for bisection. The glycosylation traits of 10 Finnish samples are compared between the new isolation method using HEK-293F cells and the old method using antigen-coated plates. **(A)** Galactosylation, **(B)** sialylation and **(C)** bisection are compared of specific IgG (upper panels) and total IgG (lower panels). The dotted line represents the equal glycosylation degree of HPA-1a-specific IgG between the old isolation method and current isolation method. Statistical analysis was performed using two-tailed paired t-test analysis comparing specific and total IgG glycosylation. Statistically significant differences are indicated by asterisks:**<0.01, ***<0.001, ****<0.0001, ns; not significant.

Supplementary figure 3: Glycosylation traits of the HIP samples. The **(A)** fucosylation, **(B)** galactosylation, **(C)** sialylation and **(D)** bisection degree of the HIP samples. The black dots indicate cases with no clinical symptoms, orange dots represent mild cases and the red dot represents the severe FNAIT case. The dotted line represents the equal glycosylation degree of HPA-1a-specific IgG and total IgG. Statistical analysis was performed using two-tailed paired t-test analysis comparing specific and total IgG glycosylation. Statistically significant differences are indicated by asterisks:***<0.001, ****<0.0001.

A

Patient sample   Capture of anti-HPA-1a   Wash and elution   Digestion and LC-MS   Data analysis

β3

αIIb

HEK-293F cells

Citric acid

Intensity

m/z

Asn297

C1q

☐ Core structure
▼ Fucose
◆ Sialic acid
● Galactose
■ GlcNAc

FcγRIIIa

B

Fucosylation (%)

p = **** (<0.0001)

Total IgG1 : Old

Total IgG1 : New

C

Fucosylation (%)

p = *** (0.0007)

Anti-HPA-1a : Old (plates)

Anti-HPA-1a : New (HEK-293F)

Y = 0.96X - 10.72

D

ns p = 0.9140

Fucosylation (%)

Old processing   New processing

A Galactosylation (%)

B Sialylation (%)

C Bisection (%)

A Fucosylation (%)
p = *** (0.0001)

B Galactosylation (%)
p = **** (<0.0001)

C Sialylation (%)
p = **** (<0.0001)

D Bisection (%)
p = **** (<0.0001)