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ORIGINAL ARTICLE

Altered Fc glycosylation of anti-HLA alloantibodies in hemato-oncological patients receiving platelet transfusions

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Abstract

Background: The formation of alloantibodies directed against class I human leukocyte antigens (HLA) continues to be a clinically challenging complication after platelet transfusions, which can lead to platelet refractoriness (PR) and occurs in approximately 5%–15% of patients with chronic platelet support. Interestingly, anti-HLA IgG levels in alloimmunized patients do not seem to predict PR, suggesting functional or qualitative differences among anti-HLA IgG. The binding of these alloantibodies to donor platelets can result in rapid clearance after transfusion, presumably via FcγR-mediated phagocytosis and/or complement activation, which both are affected by the IgG-Fc glycosylation.

Objectives: To characterize the Fc glycosylation profile of anti-HLA class I antibodies formed after platelet transfusion and to investigate its effect on clinical outcome.

Patients/Methods: We screened and captured anti-HLA class I antibodies (anti-HLA A2, anti-HLA A24, and anti-HLA B7) developed after platelet transfusions in

hemato-oncology patients, who were included in the PREPAReS Trial. Using liquid chromatography-mass spectrometry, we analyzed the glycosylation profiles of total and anti-HLA IgG1 developed over time. Subsequently, the glycosylation data was linked to the patients' clinical information and posttransfusion increments.

Results: The glycosylation profile of anti-HLA antibodies was highly variable between patients. In general, Fc galactosylation and sialylation levels were elevated compared to total plasma IgG, which correlated negatively with the platelet count increment. Furthermore, high levels of afucosylation were observed for two patients.

Conclusions: These differences in composition of anti-HLA Fc-glycosylation profiles could potentially explain the variation in clinical severity between patients.

KEYWORDS
alloimmunization, antibodies, glycosylation, HLA, platelet transfusion

1 | INTRODUCTION

Platelet transfusions are widely used for the prevention and treatment of hemorrhagic complications in thrombocytopenic patients. The success of these transfusions is usually determined by calculating the corrected count increment (CCI), which measures the increase in circulating platelets, after 1 and/or 24 h posttransfusion, by taking the total amount of transfused platelets and the patient's body surface area into account. The recurring absence of a platelet increase is known as refractoriness to platelet transfusion or platelet refractoriness (PR). This condition occurs in approximately 5%–15% of patients[1–5] with chronic platelet support and is most frequently observed in hemato-oncological patients requiring frequent platelet transfusions due to chemotherapy-induced thrombocytopenia.

Both nonimmune and immune factors are known to cause PR. The nonimmune factors are often related to the clinical condition and therapy of the patient (e.g. sepsis, fever, splenomegaly, active bleeding, medication). Immune PR occurs in approximately 20% of the PR cases and is primarily caused by alloantibodies, directed against class I human leukocyte antigens (HLA) and to a much lower degree to human platelet antigens (HPA).[6–9] The binding of these alloantibodies to donor platelets can result in their rapid clearance after transfusion, theoretically via several immunological pathways, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP).[10–16] Therefore, selecting HLA-matched donor platelets and considering recipient's HLA antibody specificity are common strategies to reduce the probability of PR.[17–20] However, because of the polymorphic nature of HLA, finding compatible donors in HLA-typed registries can be very challenging. On top of that, for yet-unknown reasons, only 30%–50% of the alloimmunized patients develop PR to unmatched platelet transfusions.[21–23] Hence, more information is needed about the underlying antibody response that might explain the differences between alloimmunized patients.

IgG contains a highly conserved N-linked glycan in its Fc region at position 297, which is essential for the antibody's function, structure, and stability. The glycan consists of highly variable extensions of a bi-antennary core structure of *N*-acetylglucosamines (GlcNAc) and mannose residues. The extensions may include a fucose, bisecting GlcNAc and up to two galactoses, each of which can be further extended by a sialic acid residue. The configuration of these sugar residues can strongly affect the antibody's effector functions and therefore the clinical course of the immune response. The lack of core fucosylation leads to an up to ~40-fold increase in binding affinity to FcγRIIIa/b, which directly translates into increased downstream effector functions, such as ADCC and ADCP.[24–30] In combination with afucosylation, galactosylation further increases the affinity to FcγRIII,[31,32] yet galactosylation is better known for its effect on complement activation.[29,33–35] Recent work has shown

Essentials

- Alloimmunization to Human Leukocyte Antigens (HLA) remains a significant complication after platelet transfusions, which can lead to immune platelet refractoriness (PR), but not all alloimmunized patients develop PR, suggesting functional or qualitative differences in HLA-specific IgG responses.
- We characterized the Fc glycosylation profile of anti-HLA Class I antibodies, developed in hemato-oncological patients after platelet transfusions, as the glycan composition can strongly affect antibody effector functions.
- The glycosylation profile of anti-HLA antibodies was highly variable between patients, especially with respect to galactosylation, sialylation and fucosylation.
- The differences in composition of anti-HLA Fc-glycosylation profiles could potentially explain the variation in clinical severity between patients receiving platelet transfusions.

that galactosylation increases the antibody's capacity to activate the classical complement pathway, through enhanced hexamerization, which consequently enhances C1q binding and CDC activity.[33,36] Sialylation has been found to slightly increase complement activation even further,[29,36,37] whereas the presence of a bisecting GlcNAc has been reported to have no effect on either complement activation nor FcγR binding.[29] Previously, we characterized the glycosylation profile of anti-HLA class I antibodies in 13 patients diagnosed with PR.[28] Although no differences were found in Fc fucosylation between anti-HLA IgG1 and total IgG1, a significant increase was observed in galactosylation and sialylation levels for approximately half of the patients.[28]

In the present study, we expanded on this pilot study by investigation a series of plasma samples from a large and well-defined patient cohort, included in the PREPAReS trial,[38–41] a randomized multicenter trial investigating the effect of pathogen reduction of platelet products on the prevention of bleeding in hemato-oncological patients, who received multiple platelet transfusions. As part of the study protocol, the clinical background of the patient, transfusion requirements and posttransfusion platelet increments were well documented. Moreover, as secondary outcome, HLA immunization was checked frequently, and plasma samples were regularly collected over a 56-day period.

In the current study, we captured anti-HLA antibodies from plasma samples in a high-throughput manner using recombinant HLA-A*02:01, -A*24:02, and -B*07:02 monomers. Using liquid chromatography-mass spectrometry, we analyzed IgG1 glycosylation profiles of total and anti-HLA IgG1 over time and linked glycosylation data to the patients' clinical information and posttransfusion increments.

2 | MATERIAL AND METHODS

2.1 | Human subjects

The plasma samples used in this study originate from the PREPAReS trial,[38–41] including hemato-oncological patients from 10 different medical centers, located in Canada, Norway, and The Netherlands. The primary aim of this study was to assess the noninferiority of pathogen-reduced-plasma-platelet concentrates (PCs) compared with plasma-PCs in terms of World Health Organization (WHO) bleeding complications ≥grade 2 during one transfusion episode.[38] Patients (aged ≥ 18 years), with chemotherapy-induced thrombocytopenia, expected to need at least two platelet transfusions, were enrolled in the study. Patients were excluded based on the following criteria: microangiopathic thrombocytopenia (HUS), immune thrombocytopenia, active bleeding with WHO bleeding grade ≥2, known presence of anti-HLA/-HPA alloantibodies or clinically relevant autoantibodies, known immune platelet refractoriness, pregnancy or lactation, indications requiring hyperconcentrated platelets, prior treatment with pathogen-reduced platelet products, or known allergy to riboflavin or its photoactive products. All patients gave written informed consent according to the Declaration of Helsinki. As part of the original study protocol, the patients' clinical background, fever, infection, mucositis, bleeding conditions, WHO performance status, the number of red blood cell/platelet/plasma transfusions, CCI at 1 and 24 h, fibrinogen levels, prothrombin time (PT), activated partial thromboplastin time (APTT), and the presence of anti-HLA class I and II antibodies were measured and documented.[38,39]

The Dutch transfusion guidelines were used as reference for the indication of platelet transfusions. The treating physician determined if or when a transfusion was ordered, as described previously.[38]

The presence of both anti-HLA class I and II antibodies was determined using the LABScreen Mixed LSM12 Screening test (One Lambda). Anti-HLA class I antibodies were determined using 12 different beads and anti-HLA class II antibodies with five beads, all beads presenting a broad array of HLA antigens. Samples were measured using a luminometer (Luminex, Luminex Corp.) and fluorescence intensities were presented relative to negative control serum provided by One Lambda as normalized background ratios (NBGs). Samples above the 3 SD cutoff (i.e., NBG ≥10.8 for HLA class I or NBG ≥6.9 for HLA class II) were considered positive.[39] The number of positive HLA beads (panel reactivity) was used as an indication of the broadness of the HLA immunization.[39]

2.2 | Production of recombinant HLA-monomers

Recombinant HLA-A*02:01, -A*24:02, and -B*07:02 heavy chains were produced in *Escherichia coli*. Peptide-HLA (pHLA) class I complexes pHLA-A*02:01, pHLA-A*24:02, and pHLA-B*07:02 were generated through *in vitro* refolding reactions with *E coli*–derived β2M, as described previously,[42] in the presence of EBV peptide GLCTLVAML (JPT Peptide Technologies GmbH), human GPR143 peptide LYSACFWWL (JPT Peptide Technologies GmbH), and RSV peptide NPKASLLSL (JPT Peptide Technologies GmbH), respectively. All pHLA class I complexes were, enzymatically biotinylated using BirA, purified by gel-filtration high-performance liquid chromatography in PBS (pH 7.4) and stored at −70°C, as reported elsewhere.[42]

2.3 | HLA-specific IgG screening ELISA

Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) were coated overnight at room temperature (RT) with 100 µl 2 µg/ml streptavidin (Thermo Fisher Scientific) in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6). After washing 4 times with 0.05% PBS-Tween 20 (PBS-T), the plates were incubated for 1 h at 37°C with 1.4 µg/ml biotinylated HLA molecules (HLA-A2, -A24, and -B7) in PBS. The plates were washed with PBS-T and incubated with 1:20 diluted plasma samples in PBS-T for 1 h at RT. Then the plates were washed with PBS-T and incubated for 1 h at RT with 1/1000 100 µl anti-IgG-HRP (MH16-1, Sanquin Reagents). After washing with PBS-T, the plates were developed with 100 µl of 0.1 mg/ml tetramethylbenzidine solution with 0.11 M Na acetate and 0.003%

H_2O_2. The reaction was terminated with 100 µl of 2 M H_2SO_4 and the absorbance was measured at 450–540 nm. Longitudinal samples were screened in antichronological order, if the most recent samples were tested positive, earlier time points were examined in a second screening phase.

2.4 | Liquid chromatography-mass spectrometry based IgG Fc glycosylation analysis and data processing

Total IgG was purified from 1 µl plasma using the AssayMAP Bravo platform (Agilent Technologies) with Protein G-coupled cartridges, as described elsewhere.[27,43] As patients were continuously given platelet transfusions with products containing plasma (35%) and storage solution (65%), there might be a small possibility of crossover of donor antibodies.

The purification of HLA-specific IgG was described previously.[44] In brief, Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) were coated overnight at RT with 100 µl 10 µg/ml streptavidin (Thermo Fisher Scientific) in coating buffer. After washing 4 times with PBS-T, the plates were incubated 1 h at 37°C with 1.4 µg/ml biotinylated HLA molecules (HLA-A2, -A24, and -B7) in PBS. The plates were washed with PBS-T and incubated with 1:2 diluted plasma samples in PBS-T for 1 h at RT. Thereafter, the plates were washed 1 time with PBS-T, 2 times with PBS, and 2 times with 50 mM ammonium bicarbonate. Elution of HLA-specific IgG was performed by the addition of 100 µl 100 mM formic acid to the samples followed by 5-min incubation on a horizontal shaking platform. The eluted HLA-specific IgG was transferred to low-binding 96-well plates, dried by vacuum centrifugation at 50°C, and subjected to overnight tryptic digestion using 10 ng/µl sequencing grade trypsin (Promega) in 40 µl 25 mM ammonium bicarbonate at 37°C.

The resulting IgG Fc glycopeptides were separated and detected with an Ultimate 3000 high-performance liquid chromatography system (Thermo Fisher Scientific) coupled to a maXis quadrupole time-of-flight mass spectrometer (Bruker Daltonics) using a CaptiveSpray and a nanoBooster, as described previously.[45] Data processing and analysis was performed in line with previous reports.[46]

2.5 | Statistical analysis

To evaluate statistical differences between the distribution of patient characteristics of the two subgroups, both χ^2 test and Mann–Whitney *U* test were used. Two-tailed/sided *p* values, and for the Mann–Whitney U test also the interquartile range (25%/75% percentile), were calculated. The statistical differences between glycan traits of HLA-specific IgG and total IgG were evaluated using the paired *t*-test with two-tailed *p* value. Statistical differences between specific glycans of HLA-specific IgG and total IgG were assessed using Wilcoxon matched-pairs signed rank test with two-tailed *p* value. To evaluate the correlation between the CCI, number of transfusions, broadness of the HLA class I immunization, anti-HLA class I titer, and anti-HLA specific glycosylation, linear regression was used, from which the goodness of fit was determined by R^2 and the 95% confidence bands were shown. All statistical analyses were performed using Graphpad Prism 8.02 (263) and the level of significance was set at $p \leq .05$. *, **, *** and **** denote a statistical significance of $p < .05$, ≤.01, ≤.001, and ≤.0001, respectively.

3 | RESULTS

To analyze the Fc glycosylation profile of HLA-specific antibodies, the patients included in the PREPAReS trial were divided into two subgroups, a group with anti-HLA class I antibodies and a group without anti-HLA antibodies (Table 1), excluding 24 alloimmunized patients with only anti-HLA class II antibodies. Based on the previously performed screening tests, as part of the study protocol, 77 patients were tested positive for anti-HLA class I antibodies, either from the start or during the study. In total, 14 patients were diagnosed with PR during the PREPAReS study, of which 12 patients presented anti-HLA class I antibodies. As expected, patients with anti-HLA class I antibodies were more frequently female and had prior pregnancies and transfusion reactions. Also, significant differences were found in the diagnosis and treatment phases. The patients who developed anti-HLA class I antibodies also showed more severe bleeding conditions, higher WHO performance status, and received more red blood cell (RBC) and platelet transfusions. Furthermore, the CCIs of the platelet transfusions and fibrinogen levels were significantly lower and longer PTs were observed for these patients compared with those without anti-HLA antibodies. Also, a larger proportion of the alloimmunized patients received pathogen-reduced platelet products.

The longitudinal plasma samples collected from these 77 anti-HLA class I positive patients, were then screened for antibodies reactive against recombinant HLA-A*02:01, -A*24:02, and -B*07:02, using a high-throughput ELISA set-(Figure 1A). This screening method resulted in overall 96 samples, originating from 35 different patients, who tested positive for antibodies reactive against at least one of the recombinant HLA monomers (Figure 1B). Seven of those 35 patients were diagnosed with PR. From all 96 plasma samples, the HLA-specific IgG fraction was purified, in parallel with the total IgG fraction, followed by the assessment of their Fc glycosylation by liquid chromatography-spectrometry (Figure 1C–E).

The systematic analysis of the Fc glycosylation alterations, comparing the major glycosylation traits of the total IgG1 and the HLA-A2 specific IgG1, revealed overall similar levels for Fc fucosylation but elevated galactosylation, elevated sialylation and decreased bisection, for the majority of the patients (Figure 2A–D and Table S1). However, it also highlighted notable exceptions and interpatient variation in glycosylation levels. For example, two patients showed considerably lower fucosylation levels compared with their total IgG1 levels (Figure 2A). These same patterns, particularly elevated galactosylation and sialylation, as observed for HLA-A2 specific

TABLE 1 Patient characteristics

Characteristic	Patients without anti-HLA antibodies	Patients with anti-HLA class I antibodies	Sign.	*p* value
Patients	465 (85.79%)	77 (14.21%)		
Age, median (IQR)	57 (49.0–63.0)	59 (49.5–65.5)	ns	.2482
Female	**138 (29.68%)**	**36 (46.75%)**	**	**.0030**
Prior pregnancies (% of female)	**93 (67.39%)**	**32 (88.89%)**	*	**.0107**
Prior RBC transfusions	318 (68.38%)	60 (77.92%)	ns	.1103
Prior PLT transfusions	281 (60.43%)	52 (67.53%)	ns	.4290
Prior transfusion reactions	**19 (4.09%)**	**8 (10.39%)**	*	**.0114**
Prior transplant procedures	27 (5.81%)	3 (3.90%)	ns	.6982
Diagnosis			*	**.0123**
Acute myeloid leukemia (AML)	210 (45.16%)	50 (64.94%)		
Acute lymphocytic leukemia (ALL)	39 (8.39%)	6 (7.79%)		
Chronic myeloid leukemia (CML)	1 (0.22%)	1 (1.30%)		
Chronic lymphocytic leukemia (CLL)	1 (0.22%)	0		
Mantle cell lymphoma (MCL)	26 (5.59%)	1 (1.30%)		
Multiple myeloma (MM)	78 (16.77%)	6 (7.79%)		
Non-Hodgkin lymphoma (NHL)	73 (15.70%)	5 (6.49%)		
Other	37 (7.96%)	8 (10.39%)		
Treatment phase			***	**.0001**
Remission induction course	187 (40.22%)	44 (58.67%)		
Consolidation course	52 (11.18%)	12 (16.00%)		
Autologous transplant	189 (40.65%)	12 (16.00%)		
Allogeneic transplant	34 (7.31%)	4 (5.33%)		
Maintenance	2 (0.43%)	1 (1.33%)		
Other	1 (0.22%)	2 (2.67%)		
Fever	246 (52.90%)	42 (54.55%)	ns	.7891
Infection	205 (44.09%)	38 (49.35%)	ns	.3896
Mucositis	208 (44.73%)	38 (49.35%)	ns	.4508
Bleeding conditions				
No bleeding	73 (13.04%)	8 (10.39%)	ns	.5134
Grade 1	314 (67.82%)	53 (68.83%)	ns	.8600
Grade 2	**254 (54.86%)**	**56 (72.73%)**	**	**.0033**
Grade 3	**8 (1.73%)**	**6 (7.79%)**	**	**.0019**
Grade 4	10 (2.16%)	1 (1.30%)	ns	.6204
Grade 2 + 3 + 4	**257 (55.51%)**	**57 (74.03%)**	**	**.0023**
WHO performance status			**	**.0003**
WHO-0	66 (14.34%)	10 (13.16%)		
WHO-1	293 (63.70%)	37 (48.68%)		
WHO-2	61 (13.26%)	16 (21.05%)		
WHO-3	16 (3.48%)	8 (10.53%)		
WHO-4	9 (1.96%)	1 (1.32%)		
WHO-5	12 (2.61%)	0		
Unknown	3 (0.65%)	4 (5.26%)		
RBC transfusions, median (IQR)	**3 (2–6)**	**5 (4–8)**	***	**.0001**
Plasma transfusions, median (IQR)	0 (0–0)	0 (0–0)	ns	.6384
PLT transfusions, median (IQR)	**4 (2–7)**	**6 (4–10.5)**	****	**<.0001**

(Continues)

TABLE 1 (Continued)

Characteristic	Patients without anti-HLA antibodies	Patients with anti-HLA class I antibodies	Sign.	*p* value
Pathogen reduction	**218 (46.88%)**	**49 (63.64%)**	**	**.0065**
Age platelet concentrate, median (IQR)	4 (3–5)	4 (3–5)	ns	.3356
CCI 1 h, median (IQR)	**13.71 (8.76–24.00)**	**8.56 (6.00–11.90)**	****	**<.0001**
CCI 24 h, median (IQR)	**6.93 (4.34–11.39)**	**5.17 (2.73–7.28)**	***	**.0007**
Fibrinogen levels, median (IQR)	**4.5 (3.7–5.3)**	**4.1 (3.5–4.9)**	*	**.0156**
PT (s), median (IQR)	12.01 (11.10–13.58)	12.50 (11.12–13.70)	ns	.4739
PT (INR), median (IQR)	**1.10 (1.05–1.20)**	**1.15 (1.1–1.23)**	*	**.0227**
aPTT (s), median (IQR)	30.50 (27.83–33.55)	29.58 (26.69–33.00)	ns	.0668

Note: Statistical significance was tested using Mann–Whitney or χ^2 tests (significant values are depicted in bold).
The level of significance was set at $p \leq .05$. *, **, *** and **** denote a statistical significance of $p < .05$, ≤.01, ≤.001, and ≤.0001, respectively.

IgG1, were also found for HLA-A24- (Figure 2E–H) and HLA-B7-specific IgG1 (Figure 2I–L). When we investigated individual glycan compositions, taking the overall glycosylation changes into account and comparing the differences between total and HLA-specific IgG1, the most prominent differences were observed as an up to 2.5-fold decrease in agalactosylated, fucosylated anti-HLA IgG1, and even more so for the nonbisected than the bisected variant (H3N4F1 [G0F] and H3N5F1 [G0FN], respectively). An up to 1.7-fold increase in fully galactosylated, nonbisected anti-HLA IgG1 was observed, both without and with one sialic acid residue (H5N4F1 [G2F] and H5N4F1S1 [G2FS], respectively) (Figure 3A and B).

Because some patients developed antibodies reactive against more than one of the recombinant HLA monomers, we were able to compare the glycosylation profile of anti-HLA antibodies between the different specificities in the same patient and plasma samples (Figure 4A–L). In general, the glycosylation profile of all anti-HLA antibodies, originating from the same plasma samples, was very similar. However, the glycosylation profile of anti-HLA A24 antibodies showed the largest differences when comparing to either anti-HLA A2 or B7 antibodies. In particular, one of the two patients presented with low fucosylation levels, indicated in gray, showed higher levels of fucosylation for anti-HLA A24 antibodies in multiple plasma samples (Figure 4A, I). Nonetheless, these fucosylation levels were still considerably lower compared with that of total IgG1.

Furthermore, as part of the original study protocol, plasma samples were collected weekly, from the start of enrollment until day 28, and also around day 56.[38] Therefore, for most of the selected patients, multiple samples were collected, which allowed us to explore the glycosylation dynamics over time (Figure 5A–O). For most patients, the glycosylation profile of the HLA-specific IgG remained similar during this relatively short time frame. However, some patients showed an increase in galactosylation and sialylation and decrease in bisection over time. These changes were most noticeable for anti-HLA A2 antibodies and were incidentally concomitant to an increase in the specific anti-HLA antibody levels (Figure 5D, G, J, and M).

Hereafter, we investigated the relationships between anti-HLA class I titer, the broadness of the HLA class I immunization and the anti-HLA class I antibody glycosylation profile, the number of transfusions and their outcome by the CCI at 1 and 24 h (Figure 6). Both the titer (signal strength) and broadness of the immunization (panel reactivity) were calculated from the data of the LABScreen Mixed LSM12 Luminex assay, as described previously.[39] The presence of anti-HLA class I antibodies impacted both the number of transfusions and the CCIs (Table 1). On top of that, a weak linear correlation was found between the anti-HLA class I titer and the number of platelet transfusions (Figure 6A) and the CCIs at 1 and 24 h (Figure 6B). Unsurprisingly, both CCIs, at 1 and 24 h, showed strong correlation (Figure 6C). Generally, the CCI at 24 h is lower than 1 h after the same transfusion. The broadness of the HLA class I immunization showed similar trends as the anti-HLA class I titer, a weak correlation was found with the number of platelet transfusions and corresponding CCIs (Figure 6D and E). This was expected to some extent, as the broadness of the HLA class I immunization is also strongly correlated to the anti-HLA class I titer (Figure 6F).

Then, we investigated the relationship between the Fc glycosylation profile of the anti-HLA antibodies and the number of transfusions or posttransfusion increment. Anti-HLA- glycosylation profiles of all specificities were combined for each patient. We focused on Fc galactosylation and sialylation levels as only two of 35 patients showed decreased fucosylation levels of the anti-HLA antibodies. These two patients received 18 or five different platelet transfusions during the study, respectively. The median CCIs of these transfusions were 4.71 or 7.02 at 1 h, and 3.35 or 5.24 at 24 h, respectively. Neither Fc galactosylation nor sialylation of anti-HLA IgG1 showed a correlation with the number of transfusions events (Figure 6G, H). However, a clear negative correlation was found with the posttransfusion increment and both Fc galactosylation and sialylation levels of anti-HLA antibodies (Figure 6I, J). No correlation was found between the antibody titer and the type of glycosylation (Figure 6K), highlighting their own predictive value. Because sialyltransferases require galactose as substrate, it was not surprising to see a strong correlation between anti-HLA IgG1 Fc sialylation and galactosylation (Figure 6L).

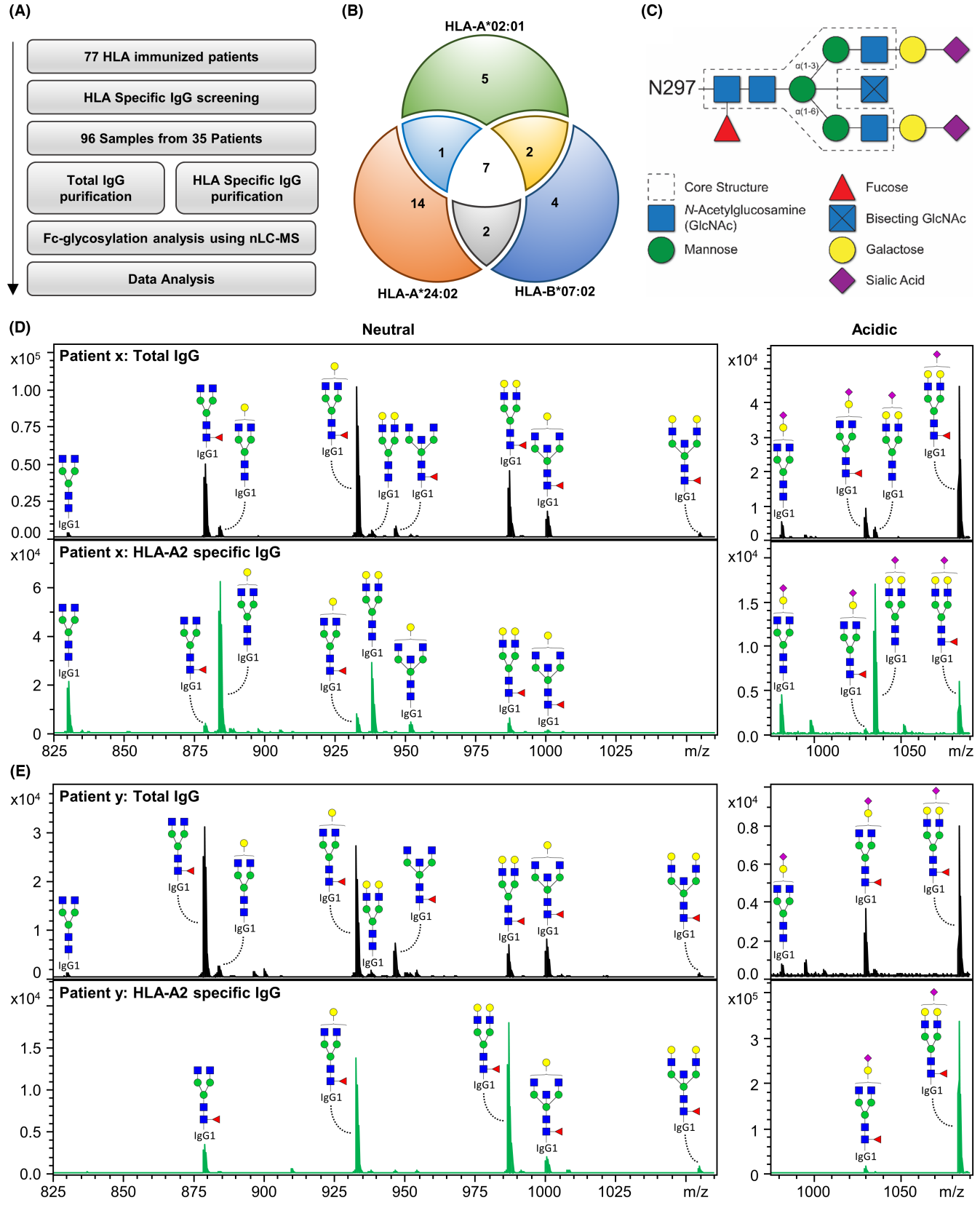

FIGURE 1 Fc glycosylation analysis of total and anti-HLA IgG1. (A) Flow chart of the entire workflow. (B) Venn diagram showing the HLA-specific antibodies shared between 35 investigated patients. (C) Depiction of the N297-linked glycan structure with symbols depicting individual monosaccharides. (D-E) Representative mass spectra illustrating the observed interindividual variability between two patients' anti-HLA-A2 specific IgG1 Fc glycosylation profile analyzed on the glycopeptide level. Left: neutral IgG1 glycopeptides; right: acidic IgG1 glycopeptides shown for both the total and the HLA-A2-specific IgG1 fraction of two patients.

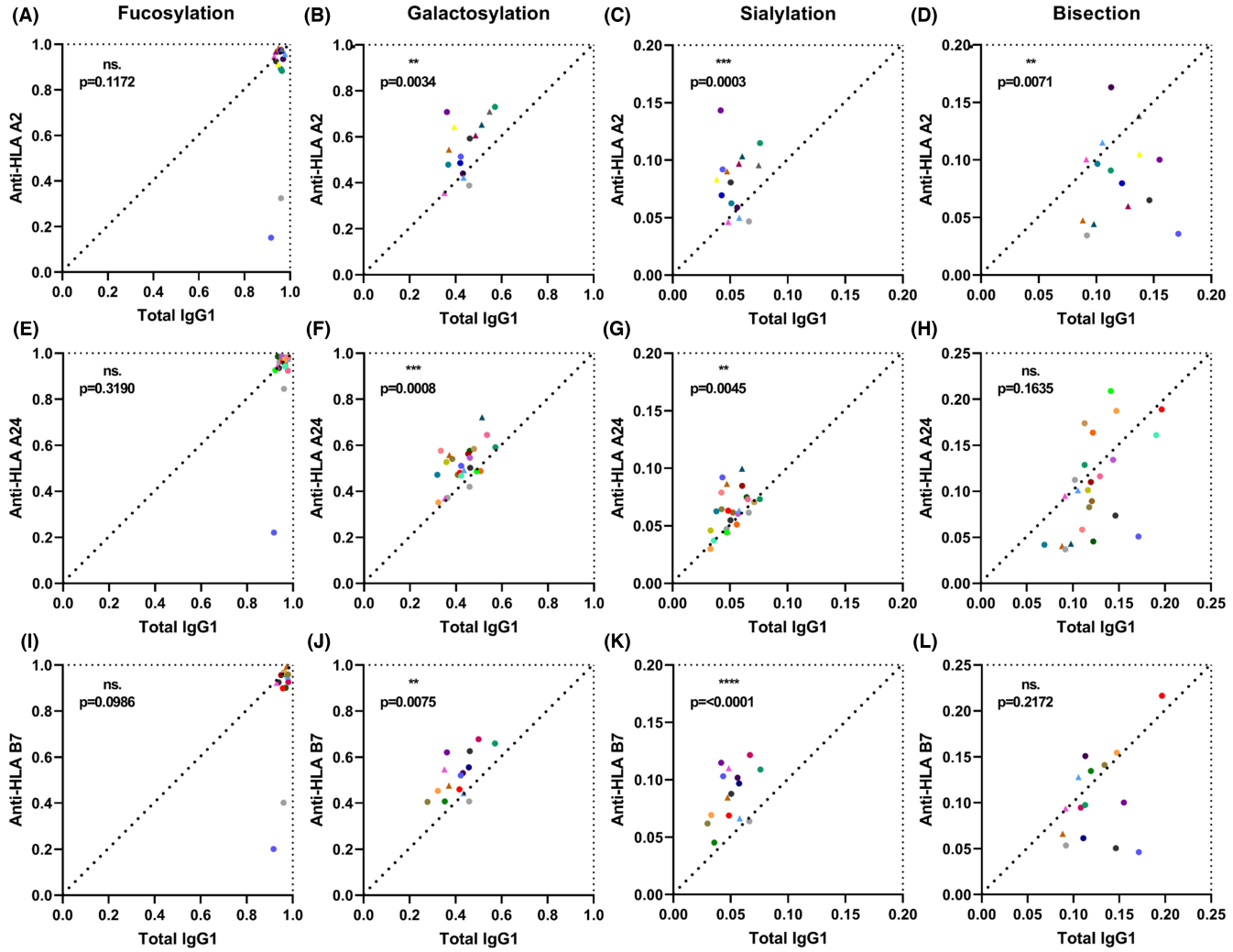

FIGURE 2 Fc glycosylation profiles of anti-HLA specific (y-axis) and total IgG1 (x-axis) for 35 patients. (A-P) The glycosylation profile was divided into the major glycan traits depicted in the columns as fucosylation, galactosylation, sialylation, and bisection. The x- and y-axes show the relative abundance of the glycan traits of either the total IgG1 or anti-HLA specific IgG1 (1.0 = 100%). Each dot represents the combined glycosylation profile of all available samples of an individual patient; patients are color-coded. Patients diagnosed with platelet refractoriness (PR) are indicated with triangle symbols. A paired *t* test with two-tailed *p* value was performed. The diagonal, dotted line represents the equal ratio between total IgG1 and the HLA-specific IgG1. Fc galactosylation and sialylation were significantly increased for anti-HLA antibodies compared with the total IgG1. Bisection was decreased for the majority of the patients and two patients showed lower levels of fucosylation of anti-HLA antibodies.

Last, we investigated the differences in alloimmunized patients who were diagnosed with PR (Figure S1). From all study participants, 14 were obliged to leave the study prematurely because of severe platelet refractoriness. Twelve patients developed anti-HLA class I antibodies, from which seven patients had either anti-HLA A2, A24, and/or B7 antibodies, of which the Fc glycosylation was analyzed. For the patients diagnosed with PR significantly lower CCI's at both 1 and 24 h were observed than the other alloimmunized patients (Figure S1A,B). The PR patients also developed a broader alloimmunization with higher anti-HLA class I titers (Figure S1C,D). In addition, although nonsignificant because of a small sample size, a larger increase in galactosylation and sialylation and larger decrease in bisection of HLA specific IgG was observed in PR patients in comparison to the other alloimmunized patients (Figure S1E–L).

4 | DISCUSSION

Alloimmunization after platelet transfusions remains a clinically challenging complication that could lead to PR, which is associated with numerous detrimental consequences including increased number of required transfusions, increased risk for bleeding,[47,48] decreased survival rate,[47] longer hospitalization and higher hospital costs,[49] particularly for patients requiring long-term platelet support (e.g., during treatment for oncological and/or hematological conditions) Anti-HLA antibodies can be formed during previous incompatible platelet transfusions, organ or bone marrow transplantations or pregnancies. Binding of these alloantibodies to donor platelets can result in rapid clearance after transfusion, suppositionally via CDC, ADCC, and/or ADCP.[10–16] Interestingly, not

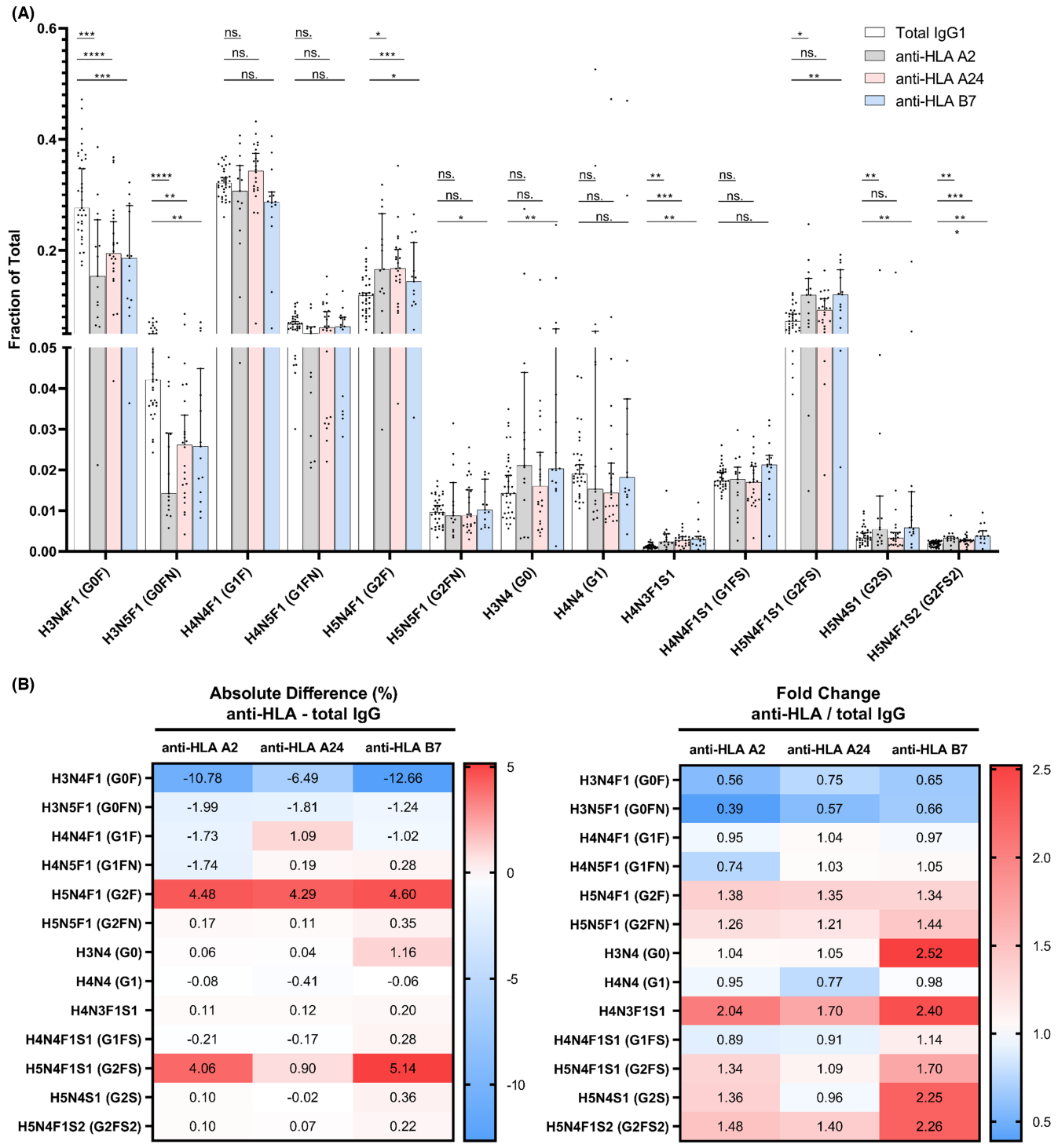

FIGURE 3 Differences in individual Fc glycan levels between HLA-specific IgG1 and total IgG1. (A) Fc glycan levels for total IgG1, anti-HLA -A2, -A24, and -B7 specific IgG1. The y-axis shows the relative abundance of the different glycans (1.0 = 100%). Bars represent the median of all patients with 95% CI. Each dot represents an individual patient. The statistical difference was calculated using the Wilcoxon matched-pairs signed-rank test with two-tailed *p* value. (B) Heatmaps of the differences in Fc glycosylation between HLA-specific IgG1 and total IgG1 on glycan level. The absolute differences were calculated using paired data by subtracting the percentages of the total IgG1 from the HLA-specific IgG1 levels per patient. The fold change was calculated by dividing the percentages of the HLA-specific IgG1 by the total IgG1 levels per patient. Values represent the median of all patients. The differences between HLA-specific IgG1 and total IgG1, were most prominently observed as a decrease in H3N4F1 (G0F) and H3N5F1 (G0FN), and an increase in H5N4F1 (G2F) and H5N4F1S1 (G2FS).

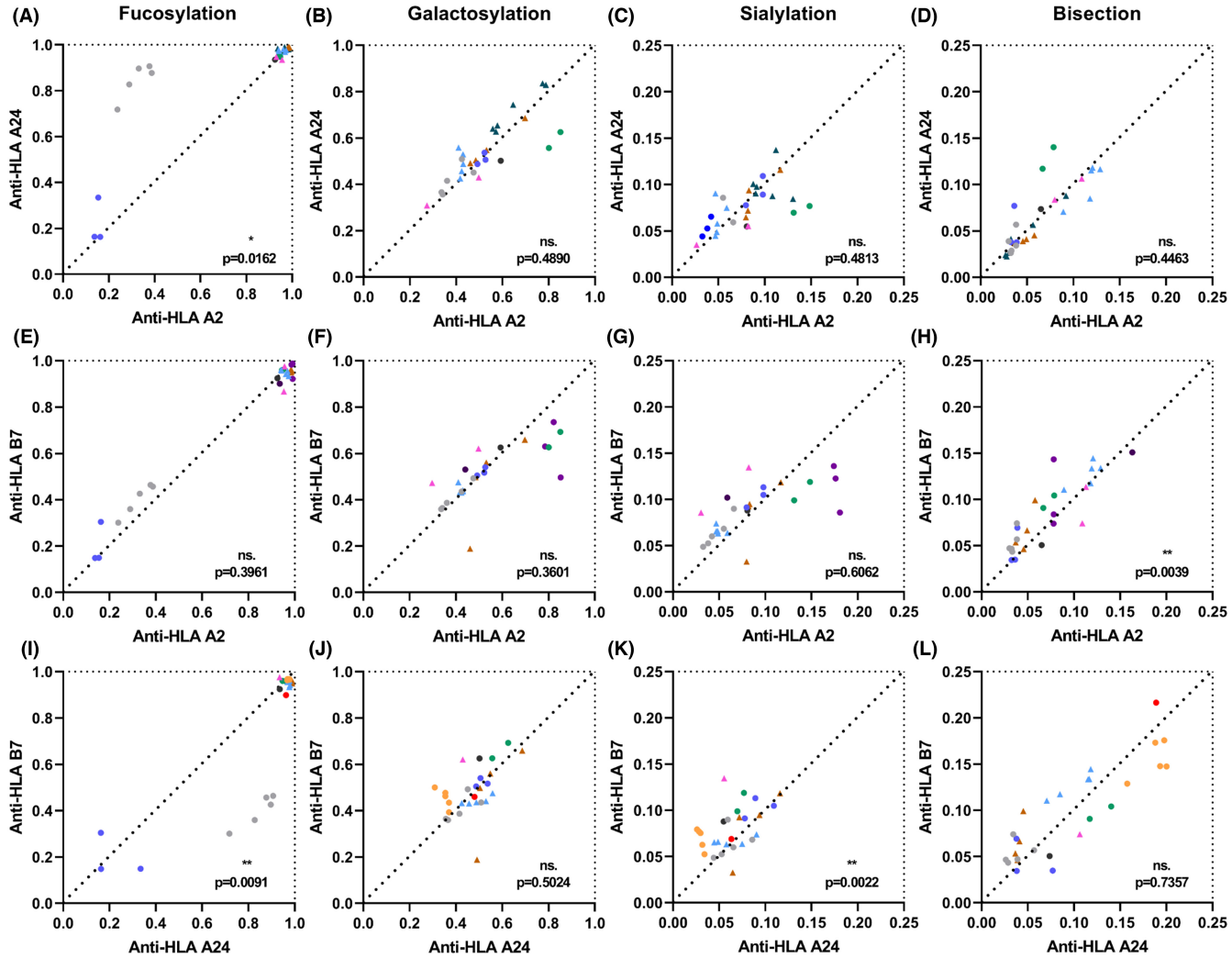

FIGURE 4 Comparison between Fc glycosylation profiles of anti-HLA specific antibodies, originated from the same plasma samples. (A-L) The glycosylation profile was divided into the major glycan traits; fucosylation, galactosylation, sialylation, and bisection. The x- and y-axes show the relative abundance of the glycan traits of the different anti-HLA specific IgG1 (1.0 = 100%). Each dot represents an individual plasma sample, patients are color-coded. Patients diagnosed with platelet refractoriness (PR) are indicated with triangle symbols. The statistical difference was calculated using the paired *t* test with two-tailed *p* value. The diagonal, dotted line represents the equal ratio between compared HLA-specific IgG1.

all alloimmunized patients develop PR to random platelet transfusions, which indicates interpatient variation and differences in HLA-specific IgG responses.

Here, we characterized the Fc glycosylation profile of anti-HLA class I antibodies, developed in hemato-oncological patients, as the glycan composition can strongly affect antibody effector functions and consequently the immune response and clinical course.[27,28,50,51] We observed a prominent increase in both galactosylation and sialylation and a decrease in bisection of HLA specific IgG1 in 30 of 35 the patients. Only two of 35 investigated patients showed high levels of afucosylation of the HLA antibodies. These results were observed for all investigated anti-HLA specificities and mostly in line with our previous observations in 13 patients diagnosed with PR,[28] with the notable exception that afucosylation of anti-HLA antibodies has not been observed earlier, most likely because of the limited size of the previously investigated cohort.

Although the majority of human IgG responses are dominated by fucosylated IgG, we and others have observed a varying degree of afucosylated IgG to red blood cell and platelet antigens in alloimmunization during pregnancy,[28,30,52] to *Plasmodium falciparum* antigens expressed on infected RBC[43] and to enveloped viruses.[27,50,53] This afucosylation increases the binding affinity to FcγRIIIa and FcγRIIIb up to 40-fold, with even stronger changes being observed in associated effector functions, such as ADCP and ADCC by myeloid or NK-cells.[26,29,43] Based on our findings, we hypothesized previously that afucosylated IgG responses are primarily targeted to antigens expressed on the membrane of host cells and not towards soluble antigens or antigens presented on pathogens.[27] Although this hypothesis seems to fit all cases in which afucosylated IgG responses have been observed, being particularly strong for infected RBC, RhD, and HPA-1a,[30,43,51,52] some RBC antigens seem to form notable exceptions (e.g., RhE and Rhc to some degree).[54] A similar situation

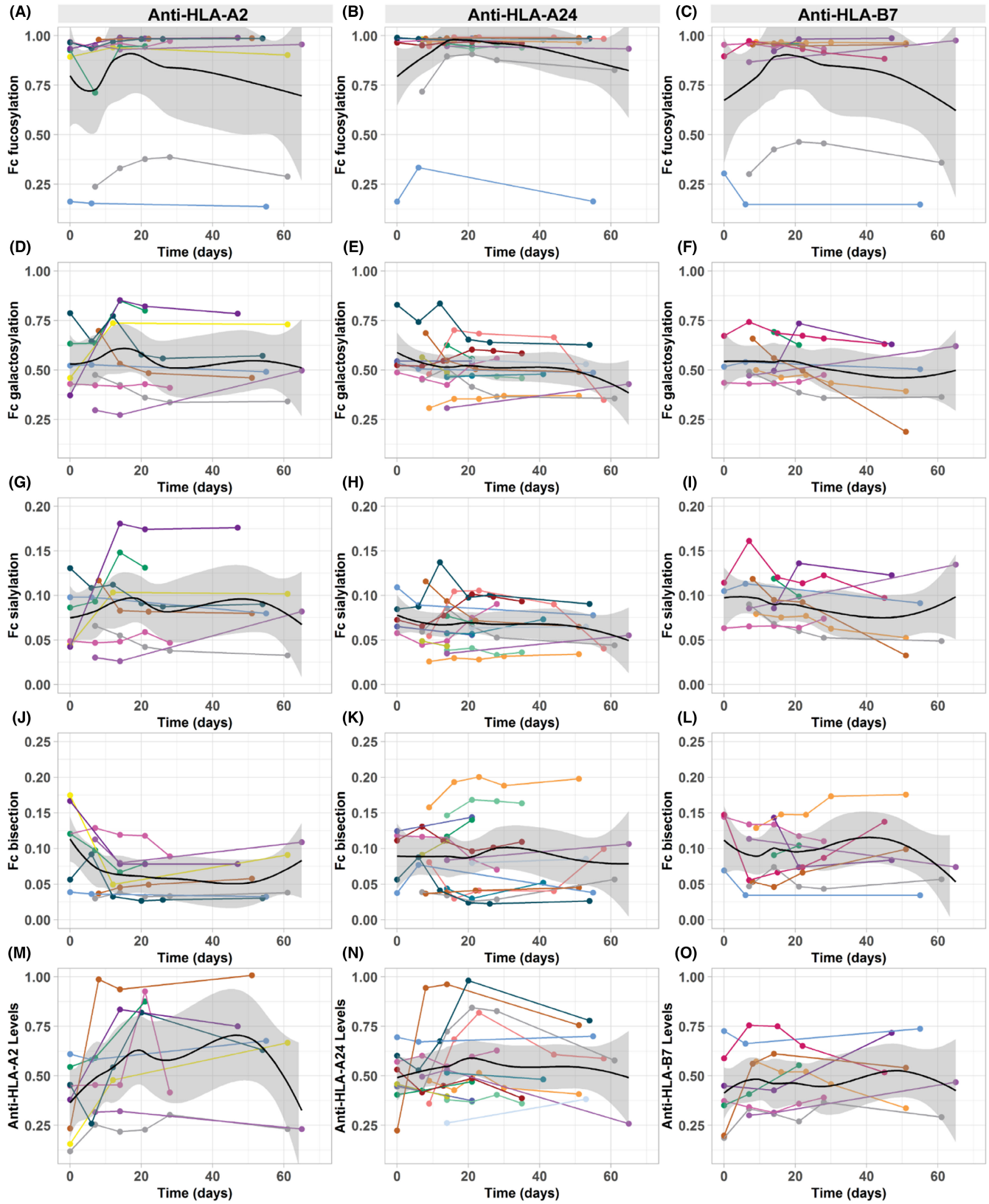

FIGURE 5 The glycosylation profile of anti-HLA specific IgG1 over time. (A–L) The glycosylation profile of HLA-specific IgG1 was divided into the major glycan traits; fucosylation, galactosylation, sialylation, and bisection. The y-axis shows the relative abundance of the different glycan traits (1.0 = 100%). Plasma samples were collected weekly, from the start of enrollment up until day 28, and at approximately day 56. Each colored line represents multiple time points of the same patient. The black line represents the mean of all patients with 95% confidence bands. (M-O) Anti-HLA specific antibody levels determined by anti-IgG Fc ELISA (absorbance, OD at 450–540 nm).

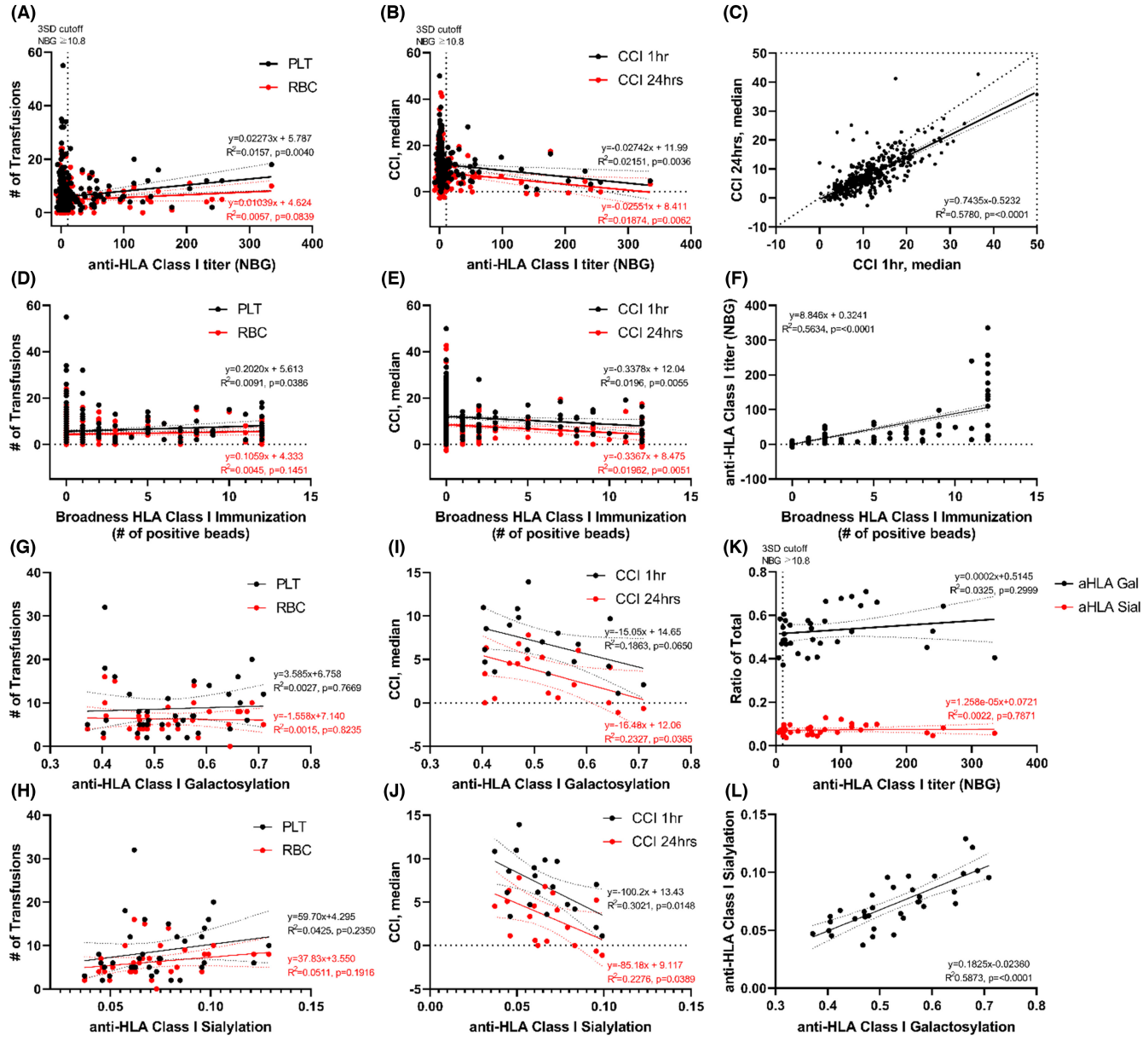

FIGURE 6 Association between anti-HLA class I antibody and transfusion parameters. The linear correlation between: (A) anti-HLA class I titer vs. number of red blood cell (RBC) and platelet (PLT) transfusions. (B) Anti-HLA class I titer vs. the corrected count increments (CCI) at 1 and 24 h. (C) CCI at 1 h vs. CCI at 24 h. (D) The broadness of HLA class . Immunization vs. number of transfusions (E). The broadness of HLA class I immunization vs. CCI (F) the broadness of HLA class I immunization vs. anti-HLA class I titer. (G) Anti-HLA class I Fc galactosylation vs. number of transfusions. (H) Anti-HLA class I Fc sialylation vs. number of transfusions. (I) Anti-HLA Class I Fc galactosylation vs. CCI. (J) Anti-HLA class I Fc sialylation vs. CCI. (K) Anti-HLA Class I titer vs. anti-HLA Class I Fc galactosylation and sialylation. (L) Anti-HLA class I Fc galactosylation vs. Fc sialylation. The titer of anti-HLA class I antibodies was determined using the LABScreen Mixed LSM12 Screening test (One Lambda). Fluorescence intensities were presented as normalized background ratios (NBG). The number of positive HLA class I beads was used as an indication of the broadness of the HLA class I immunization. The average anti-HLA class I titer and the maximum number of positive beads was used per patient. Anti-HLA class I glycosylation levels from HLA-A2, -A24, and -B7 were combined per patient. To evaluate the correlation between parameters, linear regression was used, from which the goodness of fit was determined by R^2. The 95% confidence bands are indicated with dotted lines.

seems to apply to HLA alloimmunizations, both in our present and previous studies,[28,44] the majority of patients have normal levels of fucosylated IgG, except for two patients, indicating that the membrane association seems to be necessary yet not sufficient for the induction of afucosylated IgG. As for these two patients presented with low fucosylated anti-HLA IgG, no common characteristics were found. They differed in sex, type of oncological disease, treatment phase, study arm, and in the intensity and panel reactivity of the HLA alloimmunization.

On the other hand, for the majority of the patients, both Fc galactosylation and sialylation levels were increased. These results are in agreement with previous studies showing that Fc galactosylation and

sialylation are often increased with recent or active immunization, as seen after COVID-19 infection,[27] vaccination,[55,56] and alloimmunization.[28,30] Therefore, we would indeed expect to see an increase in galactosylation and sialylation after the first and subsequent exposures to foreign HLA after incompatible platelet transfusions. Because no information was available about the HLA typing of the patient or the given platelet products, we were not able to pinpoint when patients were exposed to specific HLA. However, we did observe an increase in these glycan traits with increased anti-HLA levels in some patients.

Increased galactosylation and sialylation are both associated with increased complement activation and CDC activity.[29,33–37] Recently, we have shown that these increased levels of galactosylation and sialylation of anti-HLA hIgG1 mAbs increased complement deposition and CDC activity on human platelets.[57] We and others have reported that galactosylation increases the antibodies' capacity to activate the classical complement pathway, through enhanced hexamerization, which consequently enhances C1q-binding and CDC activity.[33,36] Furthermore, sialylation has been found to increase complement activation slightly further, albeit to a varying degree.[29,36,37] Originally, it was assumed that platelet clearance, observed after transfusion, was primarily mediated through FcγR-mediated phagocytosis. However, in agreement with our previous work on HPA-1a in FNAIT,[30] we observed that both the levels of galactosylation and sialylation were positively associated with decreased platelet count increments, which could be an indication for the involvement of the complement system. These results are in line with other studies that have shown that selecting platelet donors based on C1q-fixing solid-phase screening improve posttransfusion platelet increment[58] and that a single injection of the complement inhibitor, eculizumab, resolved PR in four of 10 patients in a small pilot trial.[59]

The PREPAReS study, from which our plasma samples originated, was not originally designed for analyzing the glycosylation profile of anti-HLA class I antibodies but for the evaluation of pathogen reduction on platelet transfusion products. Although no differences were observed in the glycosylation profile of anti-HLA antibodies between patients in both study arms, it was previously shown that pathogen reduction could affect the transfusion increments, the number of transfusions, and the risk for alloimmunization.[39] Also, a small bias can have occurred during patient selection as patients with known alloantibodies and severe immune refractoriness were not included in the study. Furthermore, no information was available about the (in)compatibility of the platelet products and only a subset of the present anti-HLA antibodies could be investigated without exploring the possible effects of loaded peptides, know to slightly affect binding of some antibodies.[60]

In summary, our data demonstrate the diversity in Fc glycosylation profiles of anti-HLA class I antibodies between patients. On the one hand, afucosylated IgG1 response was induced in two of the 35 patients, which could steer the immune response ADCC and ADCP. On the other hand, upregulation of galactosylation and sialylation levels in these antibodies, demonstrated enhanced activation of the classical complement pathway in PR, and associate with elevated platelet clearance. By characterizing these differences in IgG1 responses, our work leads to a better understanding of immune PR, which could explain the variation in both the increment of platelet transfusions and perhaps also the occurrence of transfusion reactions of HLA incompatible transfusions. However, this should be further explored in larger prospective studies in which the HLA type of the transfused platelets is known. In addition, these finding highlight that more research is required into the immunomodulatory mechanisms and pathways that induce the observed glycosylation changes.

AUTHOR CONTRIBUTIONS

Thijs L.J. van Osch, Tamas Pongracz, Jan Voorberg, Rick Kapur, Leendert Porcelijn, C. Ellen van der Schoot, Masja de Haas, Jean-Louis H. Kerkhoffs, Pieter F. van der Meer, Manfred Wuhrer, and Gestur Vidarsson designed and supervised experimental work. Thijs L.J. van Osch and Tamas Pongracz performed experiments and collected data. Dionne M. Geerdes, Juk Yee Mok, and Wim J.E. van Esch developed vital reagents. Thijs L.J. van Osch and Gestur Vidarsson wrote the manuscript, which was edited by all authors. All authors analyzed and interpreted data and approved the manuscript.

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CONFLICT OF INTEREST

No conflicts of interest to disclose.

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SUPPORTING INFORMATION

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