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ORIGINAL RESEARCH

TRANSFUSION

HLA antibodies in fetal and neonatal alloimmune thrombocytopenia

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Abstract

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by antibodies against human platelet antigens (HPA). However, in many cases that meet clinical criteria for the condition, maternal sera do not have HPA antibodies. In studies examining whether human leukocyte antigen (HLA) antibodies cause FNAIT, the results are limited and inconclusive. This study sought to examine whether clinically suspected FNAIT cases with absent maternal HPA antibodies had different HLA antibody strength and specificity compared to controls.

Study Design and Methods: A retrospective case–control study assessed class I HLA antibody strength and specificity in cases submitted for testing to Versiti, Wisconsin. There were 813 cases that met initial screening criteria, but written consent could only be obtained for 50. After review of medical records and expert panel review, 31 cases with clinical criteria of FNAIT and maternal HLA but not HPA antibodies were included. Each case was matched for maternal age, gestational age at delivery, parity, and race/ethnicity to two controls from unaffected pregnancies that had maternal serum HLA antibodies.

Results: FNAIT cases were found to have both significantly higher HLA antibody strength, measured by mean fluorescence index (MFI), and broader HLA antibody specificity at antigen epitope level, compared to matched controls ($p < .001$). p-values remained significant after controlling for parity and gestational age at delivery.

Discussion: Additional studies are needed to further examine whether the strong HLA antibodies identified in HPA-antibody-negative cases directly cause neonatal thrombocytopenia and whether prenatal treatment may be warranted in select cases to prevent recurrence.

KEYWORDS

antibodies, FNAIT, HLA, neonatal, pregnancy, thrombocytopenia

1 | INTRODUCTION

Neonatal thrombocytopenia, defined as a platelet count $<150 \times 10^9$/L, can be due to a wide array of causes, including infection, birth asphyxia, chronic state of intrauterine hypoxia, disseminated intravascular coagulation, inborn errors of metabolism, drug-related, genetic, and alloimmune.[1] Cases with a platelet count $<50 \times 10^9$/L, are associated with significant morbidity, largely due to bleeding complications, with intracranial hemorrhage (ICH) being the most serious. Evaluation for the underlying cause is typically undertaken to guide neonatal management, and usually includes testing for fetal and neonatal alloimmune thrombocytopenia (FNAIT).

FNAIT is an immune-mediated platelet disorder and a significant cause of neonatal morbidity. Clinical criteria for FNAIT include either a nadir platelet count below 100×10^9/L at birth or within 7 days after birth of the affected child and/or fetal intracranial hemorrhage, both without a clear alternative cause.[2] Even if there is an apparent cause, FNAIT should be strongly considered if the platelet count is $<50{,}000 \times 10^9$/L and especially $<20{,}000 \times 10^9$/L.[3] FNAIT is known to be caused by IgG alloantibodies that form due to maternal immunization against incompatible fetal human platelet antigens (HPA) inherited from the father. Maternal IgG HPA antibodies cross into fetal circulation leading to clinical manifestations of the disease.

Scenarios present all too commonly in which the clinical criteria for FNAIT are met, but HPA antibodies are not detected, and there are no clear alternative causes of thrombocytopenia. Such cases appear to have an immune-mediated etiology, and it is common to detect maternal antibodies against human leukocyte antigens (HLA) class I. Platelets express relatively high levels of HLA class I, and HLA antibodies have been considered as a potential cause of FNAIT.[4]

HLA class I antibodies are known to cause platelet clearance in disorders such as platelet transfusion refractoriness, and transfusion-related acute lung injury (TRALI), as well as in bone marrow and organ rejection. HLA sensitization frequently occurs not only as result of previous transfusions but also during pregnancy when the maternal immune system recognizes incompatible paternal-derived fetal HLA antigens. In fact, HLA class I antibodies are detected in up to 50% of pregnant women and higher frequencies correlate with increasing parity.[5] While HLA antibodies do not appear to be pathogenic in most pregnancies since few of the neonates are born with thrombocytopenia, there is debate as to whether HLA antibodies can cause thrombocytopenia and even ICH in rare cases.

Following a pregnancy, where clinical criteria for FNAIT are met, and only class I HLA antibodies are detected on laboratory evaluation, which is commonly seen at Versiti, an important clinical question is whether there is risk of recurrence in future pregnancies. Although there are established protocols that guide treatments for FNAIT in subsequent pregnancies and guidelines for monitoring maternal serum antibody status in suspected FNAIT when HPA antibodies are not detected,[6] there are no guidelines for managing pregnancies when only HLA antibodies are detected in maternal serum. In addition, empiric application of known antenatal FNAIT treatment strategies for suspected FNAIT based on HLA antibodies pose risks, as the treatments are not without complications.[7,8]

Over the past 30 years, there have been multiple attempts to determine if HLA class I antibodies cause FNAIT,[9–12] including case reports in which the clinical criteria for FNAIT are met, HPA alloantibodies are absent, and only HLA class I antibodies are detected in maternal serum.[13–17] However, in every study, it was unclear if the maternal HLA antibodies detected were coincidental or if they were causative of FNAIT, and results from three prior retrospective case-controlled studies did not reach a consensus.[9–11]

Therefore, in this study, we specifically examined HLA class I antibody strength, measured by mean fluorescence index (MFI), as well as disparate antigen or epitope specificity in suspected cases of FNAIT compared to matched controls to try and clarify this controversial issue. We hypothesized that class I HLA antibodies would be stronger and with disparate antigen specificity in cases of suspected FNAIT compared to matched controls.

2 | METHODS

2.1 | Selection of cases and controls

This study was approved by the institutional review board, Medical College of Wisconsin, Milwaukee, WI (IRB protocol number PRO00035577). We queried the database to identify samples in which class I HLA antibodies were detected and HPA antibodies were absent for all cases submitted to the Platelet & Neutrophil Immunology Lab (PNIL), Versiti Wisconsin from 10/1/2014 to 11/1/2020 for laboratory evaluation of suspected FNAIT. Contact information for submitting providers, when included, was obtained from requisition forms, and over 750 providers were telephoned and emailed to obtain physician contact information. Enrollng cases for this

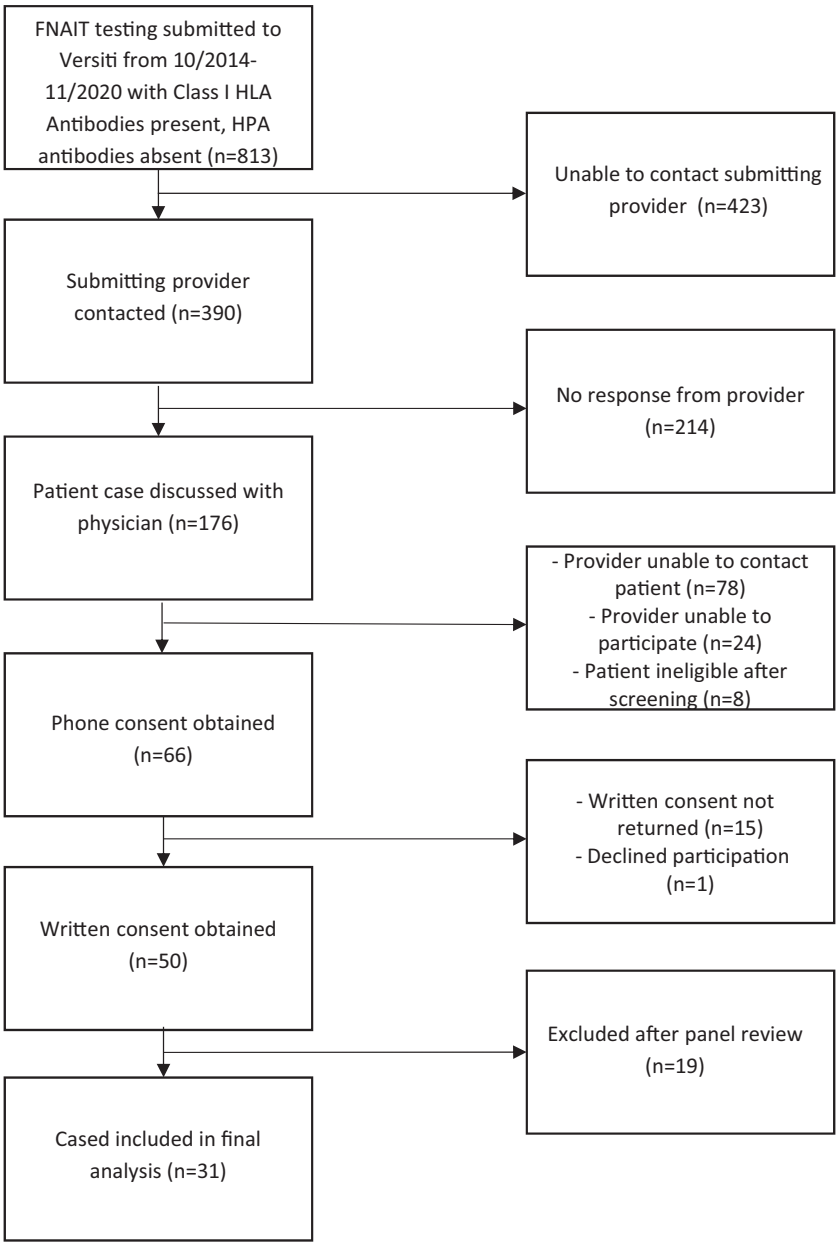

FIGURE 1 Flow diagram detailing case selection.

study was extremely challenging, which was compounded by the COVID-19 pandemic. Physicians were asked to contact the patients and obtain verbal consent for contact by us. We explained the study and obtained written consent and request for medical records. One examiner abstracted the medical records and provided the relevant clinical information to the expert panel, who was blinded to any identifying information for cases to avoid selection bias. Exclusion criteria included clear alternative non-alloimmune causes of neonatal thrombocytopenia, inability to obtain medical records, or maternal receipt of blood transfusion prior to the pregnancy examined by this study. The clinical criteria for inclusion were a neonate with nadir platelet count $\leq 100 \times 10^9$/L within 7 days of birth, and/or fetal ICH. Routine platelet counts are not standard of care in the United States, but neonatal platelet counts were obtained if there was suspicion for thrombocytopenia on physical exam. An expert panel composed of pediatric hematology and maternal fetal medicine physicians, as well as platelet immunology experts, convened to ensure cases met clinical criteria for FNAIT without alternative causes for neonatal thrombocytopenia or ICH.

Controls were obtained from the Medical College of Wisconsin Maternal Research Placenta & Cord Blood Bank. Maternal blood samples for controls were collected following signed consent during pregnancy, which typically occurred between 24–28 weeks gestation at the time of screening for gestational diabetes. Clinical information was available for each maternal control subject. Two

control subjects were paired with each case and priority for matching was in the following order: parity, gestational age at delivery, maternal age, and race/ethnicity. Controls meeting these criteria were included if they also tested positive for HLA class I antibodies. Clinical exclusion criteria for controls were any evidence of intrauterine infection, maternal thrombocytopenia, small for gestational age infant, or hypertensive disease of pregnancy. Thirty-one cases meeting clinical and serologic criteria were available for study (Figure 1), which resulted in 62 controls included for analysis.

2.2 | Laboratory testing

Laboratory testing of cases and controls was performed at Versiti, Wisconsin. Cases were tested for HPA-specific IgG antibodies and class I HLA antibodies using intact platelets and flow cytometry, and by a monoclonal antibody glycoprotein capture assay platelet antibody bead array (PABA).[18] Maternal sera were tested by PABA against both a panel of five different group O donor platelets for antibodies against HPA-1, −2, −3, −4, −5 and class I HLA, and a crossmatch against paternal platelets to detect antibodies against low frequency and new HPA. A CD109 monoclonal antibody immobilization of platelet antigens (MAIPA) assay was performed to detect HPA-15 antibodies. Genotyping of maternal, and when possible paternal blood, was performed for HPA-1 to −6, −9, and −15. Cases in which maternal sera tested positive for HLA class I antibodies and negative for HPA-specific IgG antibodies were then tested for HLA-A and HLA-B antigen specificity using the LABScreen Single Antigen HLA Class I – Combi kit (One Lambda/Thermo Fisher Scientific, Los Angeles, CA) with acquisition by a Luminex LABScan3D (One Lambda) and data analysis using HLA Fusion software (One Lambda). HLA antibody specificities were assigned based on manufacturer's instructions and lab experience using multiple criteria for positivity including mean fluorescence intensity (MFI) values, background fluorescence levels, individual bead performance, epitope patterns and cross-reactivity. An MFI value of >1000 was the general cut-off for class I HLA-A and HLA-B positive antibody results. No HLA or HPA antibody testing was performed on newborn samples, and HLA genotyping was not performed on maternal, paternal, or newborn samples.

2.3 | Statistical analysis

All statistical analyses were conducted using R Statistical Software (v4.2.0; R Core Team 2022)[19] and included Wilcoxon rank sum test, Pearson's Chi-square, Fisher's exact test, and Wilcoxon rank-sum (Mann–Whitney U test) test as appropriate. Logistic regression, adjusting for parity and gestational age at delivery, was performed. False discovery rate adjustment was performed when examining antibody specificity to help control false positive rate when performing multiple hypotheses, which was present when examining allele level antibodies. Using the Wilcoxon rank sum test, the specificity and strength/reactivity of HLA class I antibodies in the cases were compared to matched controls. HLA class I antibody specificity was analyzed by comparing the proportion of participants with HLA antibody positive reactivity (MFI > 1000) against each antigen in sera from cases vs. controls. The strength/reactivity of HLA class I antibodies was analyzed by comparing the maximum MFI signal against a single HLA antigen/bead in the cases compared to the controls.

3 | RESULTS

3.1 | Study population

Figure 1 outlines the case selection process for the study. A total of 813 samples met initial criteria during the study period. Submitting providers for 390 samples were successfully contacted with 176 physicians responding and reviewing the participant information with the study team. Of the 176 physicians successfully contacted, 78 were unable to reach the family, 24 declined to participate, and 8 cases were deemed ineligible as clinical history was not consistent with possible FNAIT. In total, 66 participants were contacted by telephone and gave verbal consent to participate, 50 written consents were obtained, and after review of medical records and discussion by the expert panel, 31 cases were selected for study.

Cases and controls were not significantly different ($p > .05$) for maternal age, gestational age at delivery, or parity of the affected pregnancy (Table 1). In total, 42% of pregnancies were primiparous for cases and controls, and the majority (74%) of cases identified as White, followed by Hispanic, Black, and Asian. There was a higher percentage of White/Caucasian women in the control group and fewer Black, Hispanic, and Asian women compared to cases ($p = .009$).

Table S1 details the clinical characteristics of the infants for cases. In each of the 31 cases, the expert panel noted that the newborn platelet counts could not be explained solely by fetal or maternal causes, and thus, was concerning for FNAIT. Nadir neonatal platelet counts were available in 29/31 cases, with a median of 26×10^9/L (IQR 11–34 $\times 10^9$/L). Two neonatal platelet

TABLE 1 Characteristics of cases and controls.

Characteristic	Cases ($n = 31$)	Controls ($n = 62$)	p-value
Age[a]	31 (28,35)	30 (28,34)	.6
Gestational Age at Delivery (weeks)[a]	39 (38,39.5)	39 (38,39)	.5
Parity[a]	2.00 (1,2)	2.00 (1,2)	>.9
Primiparous[b]	13 (41.9)	26 (41.94)	>.9
Race/Ethnicity-no. (%)[b,c]			
White	23 (74.19)	58 (93.55)	.009
Black	2 (6.45)	3 (4.84)	
Hispanic	4 (12.90)	0 (0)	
Asian	2 (6.45)	1 (1.61)	

[a]Median (IQR).
[b]Number (%).
[c]Race/ethnicity was self-reported.

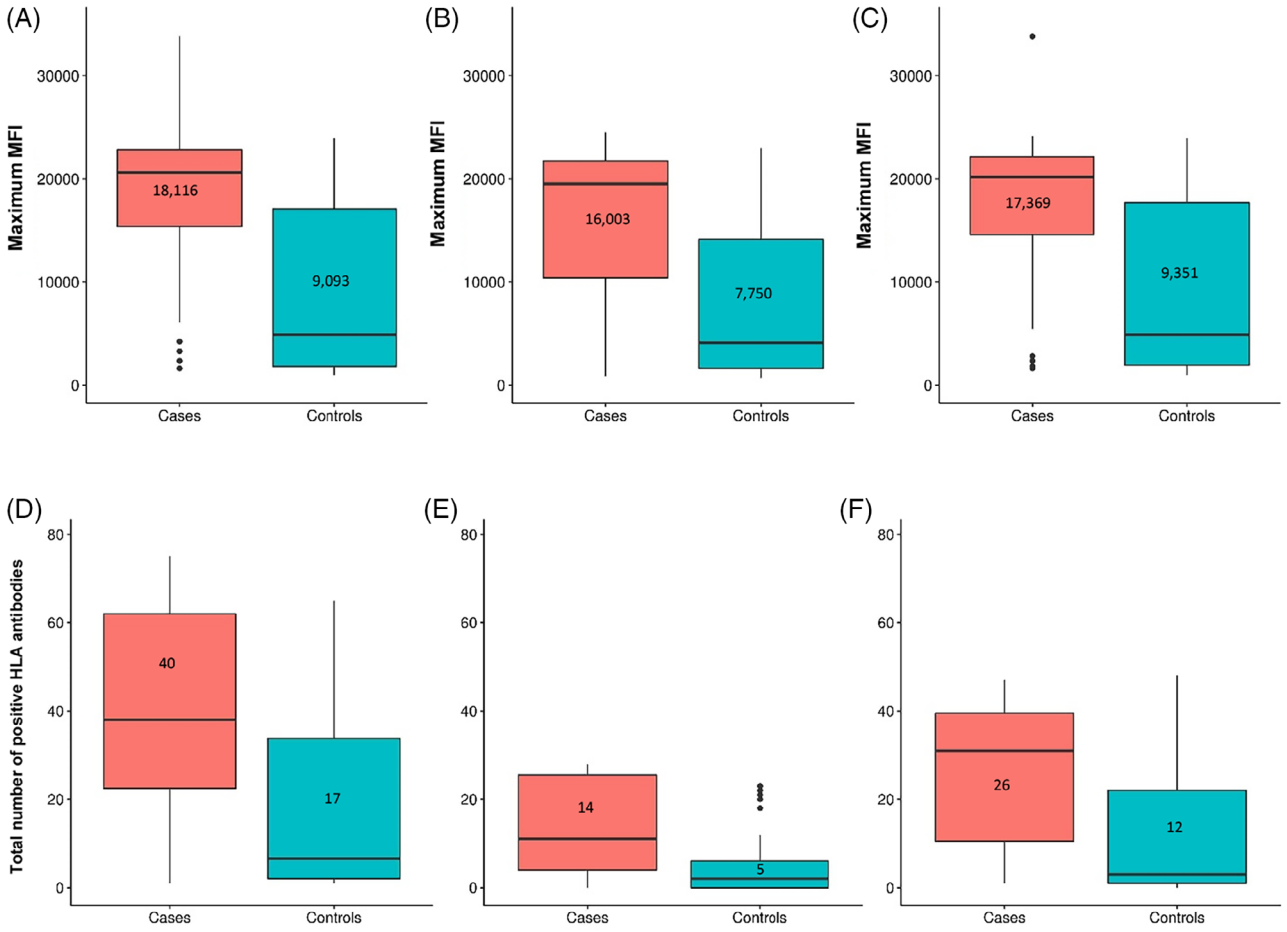

FIGURE 2 Figures 2A–C show HLA class I antibody strength depicted as mean fluorescence intensity (MFI) from Luminex assay comparing cases to controls (2A: HLA-A and B combined, and 2B: HLA-A alone, and 2C: HLA-B alone). The strength/reactivity of HLA class I antibodies is the maximum MFI signal obtained with maternal serum against a single HLA antigen/bead in the cases compared to controls. Cases had statistically higher ($p < .001$) maximum MFI compared with controls for each group. p-values remained statistically significant after controlling for parity and gestational age at delivery. Figures 2D–F show total maternal antibodies detected against various HLA antigens comparing cases to controls (2D: HLA-A and HLA-B combined, 2E: HLA-A alone, and 2F: HLA-B alone). Cases had statistically higher reactivity in all three groups, measured by total number of positive antibodies called compared with matched controls ($p < .05$). Mean values for each group are included in the box plot.

counts were not available due to neonatal demise shortly after delivery from ICH.

3.2 | HLA antibody strength in cases vs. controls

Overall strength of class I HLA antibodies (HLA-A and HLA-B) measured by MFI was significantly higher ($p < .001$) in cases compared to controls, as cases had a median of 20,613 (IQR 15,391, 22,816) compared to controls 4867 (IQR 1812, 17,074) (Figure 2A). When examining only class I HLA-A, cases also had a significantly higher maximum median MFI of 19,514 (IQR 10,404, 21,729) compared to controls 40114 (IQR 1629, 14,129) (Figure 2B). When examining only class I HLA-B, cases also had a higher maximum MFI, as cases had a median of 20,185(IQR 14,590, 22,144) compared to controls, median 4867 (IQR 1924,17,694) (Figure 2C). Comparisons remained significant for each group after controlling for parity and gestational age after delivery ($p < .001$). Figure S1 depicts maximum MFI at the epitope level for HLA-A and HLA-B in cases compared to controls.

3.3 | Allele-specific HLA antibody reactivity

Using Fisher's Exact Test, 65/81 (80.2%) of all allele level HLA class I antibodies (HLA-A and HLA-B combined) were detected at statistically higher frequency ($p < .05$) in cases compared to controls, measured by total number of positive antibodies (Figure 2D), even after performing a false discovery rate adjustment (64/81, 79.0%). Similar results were observed when analyzing HLA-A (Figure 2E) and HLA-B (Figure 2F).

3.4 | No association of HLA class I antibody strength and nadir neonatal platelet counts

We found no association between the strength of class I HLA antibodies, measured by MFI, and nadir neonatal platelet count, shown in Figure 3 (Spearman correlation $r = -0.17$, $p = .40$).

4 | DISCUSSION

Our study showed a significant difference in both the strength and range of HLA antigens recognized by maternal HLA class I antibodies in maternal sera from pregnancies meeting the clinical criteria for FNAIT, but absent HPA antibodies, compared to matched controls with uncomplicated pregnancies and unaffected infants. These findings were consistent with both of our hypotheses that class I HLA antibodies would be stronger and with disparate antigen specificity in cases of suspected FNAIT compared to matched controls.

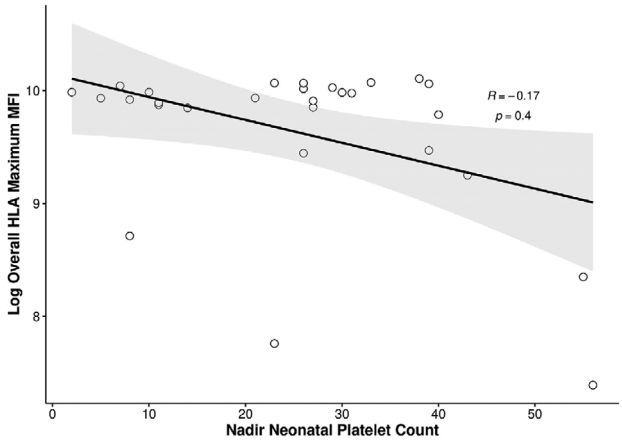

FIGURE 3 Comparison of neonatal nadir platelet counts and maximum HLA class I antibody mean fluorescence intensity (MFI) values of maternal sera for 29 cases. In 2 cases, neonatal platelet counts were not available due to neonatal demise shortly after delivery.

The diagnostic approach to a neonate with thrombocytopenia considers gestational age, birth related factors, pregnancy complications, timing of onset of thrombocytopenia, and the overall clinical status of the newborn.[20] By reviewing medical records for each mother and child pair for cases, and utilizing an expert panel to discuss each case, we developed a cohort of cases in which the neonates appeared generally healthy without obvious signs of non-immune causes of neonatal thrombocytopenia, including sepsis, necrotizing enterocolitis or other infectious etiologies. In addition, all infants in our study had platelet counts $<60 \times 10^9$/L, and all but two had a platelet count $<50 \times 10^9$/L. Three infants had chromosomal abnormalities (trisomy 21, mosaic monosomy X, and mosaic tetrasomy 12 p), however, inherited thrombocytopenia rarely presents with severe thrombocytopenia in the newborn period,[20] nor is it the sole etiology for severe thrombocytopenia.[21] As a result, the cases represent pregnancies with neonates having severe thrombocytopenia and/or ICH that appeared to have an alloimmune cause and evaluation for FNAIT was undertaken.

Maternal serologic results showed a significant and substantial difference in the number and strength of HLA antibodies present in sera from cases compared to controls. However, we found no correlation between

these metrics and nadir neonatal platelet counts. The rationale for this remains unclear. Previous work has demonstrated the presence of HLA antibodies in umbilical cord blood samples, proving that HLA antibodies cross the placenta with the potential to bind and clear fetal platelets.[12] One possibility is that only the strongest HLA antibodies are capable of triggering FNAIT. However, we were unable to determine a threshold HLA antibody MFI above which a stronger suspicion of FNAIT is more compelling. It is worth commenting that even in cases of HPA-incompatibility resulting in FNAIT, there is no clear correlation of antibody strength and neonatal platelet count.

Previous studies on the relationship between HLA class I antibodies and FNAIT have yielded conflicting results, partially due to the heterogeneity in criteria for selection of case and control groups, study methodology, and HLA antibody detection methods used. A study utilizing very similar HLA antibody test method and case and control selection as we did, also found that HLA class I antibodies from cases had significantly higher MFI compared to controls, but in contrast, they observed no difference in HLA antibody reactivity patterns and a slight inverse correlation between MFI and neonatal platelet count.[11] In that study, there was also a significant number of infants with ICH, although it was not clear that ICH was caused by HLA antibodies in fetal circulation. A study by Refsum et al coincidentally used the same HLA antibody MFI cut-off (≥1000) as in our study and like our results found higher levels of HLA antibodies in cases compared to controls.[10] In a recent study, Sachs et al examined the effect of class I HLA antibodies in cases of known FNAIT caused by HPA-1a antibodies.[9] They concluded that there was no association between class I HLA antibody presence and neonatal platelet count, ICH, or birth weight; however, this study did not examine the strength of HLA antibodies in their cohort.

It is difficult to determine how to best use MFI to analyze the potential impact of HLA antibodies on fetal platelets. The transplant field is most experienced with establishing and applying MFI obtained from HLA antibody test results.[22] MFI is based on the relative amount of IgG antibodies bound to HLA antigens attached to solid-phase beads in the assay. This method is very sensitive but has limitations. It is an in-vitro examination of the HLA antibody reactivity, which may not necessarily correlate with the in-vivo effect of the antibody on fetal/neonatal platelets. In transplant medicine, HLA antibody MFI is not used alone, but in conjunction with patient HLA type, alloimmunization history, and epitope specificities to make clinical decisions.[22] We had information regarding potential alloimmunization history but did not have HLA typing of the mothers or the affected infants, and thus, we used best available information concerning HLA antibodies available to us.

Our results combined with two previous studies,[10,11] make three studies now showing that sera from women who delivered infants with significant thrombocytopenia, and no HPA antibodies, have HLA class I antibodies that give stronger and broader reactivity when compared to HLA antibodies in sera from mothers that delivered infants without thrombocytopenia. Although these results do not prove that stronger and/or more broadly reactive HLA antibodies cause FNAIT, this correlation does deserve consideration. In suspected cases of FNAIT in which HLA antibodies are detected in maternal sera, consideration could be given to treating women with IVIG in subsequent pregnancies, particularly if the current sibling had an ICH or in the presence of "exceptionally strong" HLA class I antibodies in maternal serum and prior delivery of an infant(s) with platelet count $\leq 20 \times 10^9$/L. If the previously affected infant(s) had a platelet count $>20 \times 10^9$/L, then performing a platelet count on the baby at delivery may be sufficient. The definition of "exceptionally strong" HLA antibodies must be determined by individual referral laboratories with experience testing cases of suspected FNAIT.

Strengths of our study include thorough serologic testing for HPA and HLA antibodies performed by an experienced reference laboratory evaluating FNAIT cases, case selection using an expert panel comprised of pediatric hematology and maternal fetal medicine physicians, as well as platelet immunology experts to ensure cases met clinical criteria for FNAIT without clear alternative causes for neonatal thrombocytopenia or ICH. Lastly, our study matched cases to controls, allowing for a robust analysis.

Our study also had several weaknesses. While our cases were well-characterized both clinically and serologically the total was only 31. This was largely due to challenges in contacting submitting providers and reaching families for whom the FNAIT testing was submitted. HLA typing and obtaining blood counts of the mothers, infants, and fathers would have allowed for the most robust analysis. However, a previous study convincingly showed that almost all maternal HLA class I antibodies recognize mismatched paternal antigens inherited by the infants.[11] While we did a robust analysis of each case, we could not fully ascertain that the cause of neonatal thrombocytopenia or ICH in each case was solely due to alloimmune causes and no other factors. In addition, we did not have access to neonatal platelet counts or additional records following selection of controls so we cannot definitively say they did not develop thrombocytopenia. However, the 5th percentile for platelet counts in late preterm and term infants is 123×10^9/L,[23] thus the

likelihood of neonatal thrombocytopenia, especially $<50 \times 10^9$/L was very low. Finally, we were not able to accurately determine the timing of maternal blood sampling for cases and controls, which could have resulted in variance of HLA antibody results.

Despite these limitations, our study demonstrated important findings for families facing possible FNAIT. A future larger prospective study may be informative to confirm our observations, however, given the low incidence of this disorder, it would be difficult to conduct in a single center, as our study utilized the largest referral center for FNAIT testing in the United States. A systematic international registry of multiple centers to collect clinical information of all patients meeting criteria for FNAIT, including laboratory data, could be utilized to perform such a study in the future. Future studies should focus on the extent to which HLA antibodies enter fetal and neonatal circulation, and if under specific clinical conditions, "exceptionally strong" IgG HLA class I antibodies are causative of FNAIT. Future studies should also examine maternal, paternal, and infant HLA antibody type, in addition to HLA antibody strength and specificity, to further determine potential effects of HLA antibodies on FNAIT. Additionally, it would be beneficial to determine the recurrence rate of this clinical scenario in cases where clinical criteria of FNAIT are met and only HLA antibodies are present on laboratory evaluation, to guide management in subsequent pregnancies.

5 | CONCLUSIONS

In conclusion, we found that in suspected cases of FNAIT in which HPA antibodies were absent, HLA class I antibodies had stronger reactivity and broader specificity compared to matched controls from unaffected pregnancies. While this study contributes important information about a challenging maternal, fetal, and neonatal clinical scenario, these findings do not allow us to definitively conclude that HLA class I antibodies cause FNAIT. Future studies need to further evaluate the role HLA antibodies play in causing neonatal thrombocytopenia and intracranial hemorrhage and to determine whether prenatal treatment may be warranted in select cases to prevent recurrence.

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CONFLICT OF INTEREST STATEMENT

BRC is a consultant for Rally bio and Argenx. RFG is a consultant for Agios and Sanofi and has research funding from Novartis, Sobi, and Agios. All other authors have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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