Full Length Article

Adding to the complexity of fetal and neonatal alloimmune thrombocytopenia: Reduced fibrinogen binding in the presence of anti-HPA-1a antibody and hypo-responsive neonatal platelets

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ABSTRACT

Background: In fetal and neonatal alloimmune thrombocytopenia (FNAIT), maternal alloantibodies directed against paternally-derived platelet antigens are transported across the placenta to the fetus, where they may cause thrombocytopenia. The most serious complication of FNAIT is an intracranial hemorrhage (ICH), which may cause death or life-long disability of the child. Apart from alloantibody-mediated platelet destruction, the clinical outcome in FNAIT may be affected by properties of neonatal platelets and possible functional effects on platelets caused by maternal alloantibodies.

Methods and results: The function of umbilical cord blood platelets was compared with adult platelets in two assays, impedance aggregometry (Multiplate) and rotational thromboelastometry (Rotem). Both revealed a decreased in vitro neonatal platelet function compared to adult platelets. Consistent with this finding, activation using TRAP revealed less pronounced changes in the expression of CD62P, PAC-1, CD41 and CD42a in umbilical cord blood platelets compared to adult platelets. Furthermore, a monoclonal anti-HPA-1a antibody, derived from an immunized mother of two children with FNAIT, blocked fibrinogen binding to resting and activated umbilical cord blood and adult HPA-1aa and HPA-1ab platelets, interfered with platelet activation by TRAP, and impaired the function of umbilical cord blood HPA-1aa platelets in rotational thromboelastometry.

Discussion and conclusions: Reduced fibrinogen binding in the presence of anti-HPA-1a antibodies may disturb the neonatal hemostatic balance, characterized by poorly responsive platelets. This effect may operate in parallel to platelet destruction and contribute to the clinical outcome in FNAIT.

1. Introduction

In fetal and neonatal alloimmune thrombocytopenia (FNAIT), maternal alloantibodies target paternally-derived human platelet antigens (HPAs) expressed on fetal platelets, mediating thrombocytopenia. In Caucasians, FNAIT is most often due to anti-HPA-1a antibodies [1], and the most feared complication is a severe bleeding, in particular an intracranial hemorrhage (ICH). Not all mothers with anti-HPA-1a antibodies give birth to a thrombocytopenic child, however, and platelet

counts, the presence of fetal bleeding and maternal antibody levels are not always correlated [2–4]. This indicates, together with the clinical heterogeneity, that several factors determine the outcome of FNAIT.

The mechanisms contributing to low platelet counts and increased risk of bleedings in neonates are probably manifold. In vitro studies have suggested that anti-HPA-1a antibodies can mediate phagocytosis of platelets by monocytes [5,6], suppress fetal megakaryopoiesis [7], and that certain subtypes of anti-HPA-1a antibodies, associated with the presence of ICH, reduce endothelial cell proliferation and angiogenesis

[8]. Furthermore, maternal anti-HPA-1a antibodies may vary not only by titer or subtype, but also composition: A reduced core fucosylation of IgG-associated glycans in FNAIT has been associated with enhanced phagocytosis by monocytes and worse clinical outcome [9]. The degree to which these different mechanisms play a role *in vivo* is uncertain. In addition to the antibody aspect, the target epitope expression, the platelet HPA-1a antigen, could potentially vary. Neonates affected by FNAIT will be heterozygous for the immunizing HPA-1a antigen, but how the HPA-1a expression further differs has not been described.

Beyond the immunological aspects, fetal platelet function itself could be a determining factor in FNAIT. There are a limited number of studies on healthy neonatal platelet function, but the majority of those suggest a decreased *in vitro* platelet function in neonates, which is functionally balanced *in vivo* by distinct properties of the neonatal platelet, which matures with age [10,11]. This neonatal hemostatic balance could potentially be disturbed by a low platelet count caused by anti-HPA-1a antibodies. Moreover, the antibodies target the HPA-1a epitope, located on the fibrinogen receptor, which is crucial for platelet activation and aggregation [12]. Blocking of fibrinogen binding could therefore contribute to the disruption of neonatal hemostatic balance and increase risk of bleedings. Though rare, bleedings in FNAIT can occur early in gestation, are often severe, and may present with extremely low and relatively high platelet counts [13], which justifies a closer investigation of the platelet role in FNAIT.

1.1. Aim

The aim of this study was to investigate neonatal platelet function compared to adult platelets, and to test how the presence of anti-HPA-1a antibodies could potentially affect platelet function through binding to the fibrinogen receptor.

2. Material and methods

2.1. Study population and blood sampling

For comparison of platelet glycoprotein expression and *in vitro* platelet function between adults and neonates, umbilical cord blood samples from 12 healthy term deliveries were consecutively collected from placentas immediately after delivery and were compared with peripheral blood collected from 12 healthy adult blood donors. All samples were phenotyped for HPA-1a by flow cytometry and genotyped using an in-house RT-PCR method. Blocking experiments with monoclonal anti-HPA-1a (26-4) and fibrinogen binding was performed in two sessions: In the first, whole blood from HPA-1a typed adult donors was used (n = 3 to 4 for each genotype), in parallel to umbilical cord blood from 3 neonates collected into bags containing Citrate Phosphate Dextrose Solution (CPD) buffer. In the second session, 24 umbilical cord blood samples and 10 adult peripheral blood samples were collected and analyzed. Apart from the three first umbilical cord blood samples, all blood was collected into 3.2% sodium citrate tubes (BD Vacutainer), after the first 2 mL had been discarded. The Multiplate analyses, Rotem analyses, and flow cytometry stainings were all performed within 2.5, 4, and 6 h from blood drawing, respectively. Blood counts were measured using Medonic CA620 (Boule Medical AB). The study was approved by the regional ethics review board in Stockholm (approval no. 2015/2037-32).

2.2. Whole blood flow cytometry staining protocol for platelet markers

Flow cytometry was performed to analyze the platelet expression of CD61 (GPIIIa) derived from the HPA-1a allele, to visualize the expression of the HPA-1a epitope on platelets (from here on referred to as CD61/HPA-1a). Glycoprotein GPIIb (CD41) was also analyzed, representing the other part of fibrinogen receptor dimer (GPIIb/IIIa). In addition, glycoprotein GPIX (CD42a), and activation marker P-selectin

(CD62P) were measured to further visualize activation-induced changes. Citrated whole blood was diluted with modified HEPES/ Tyrode's buffer 1:50, and stained for 15 min in the dark at room temperature (RT) with the following antibodies: Anti-CD42a (eFluor 450, GR-P clone, eBioscience/Affymetrix), anti-CD62P (APC, AK4 clone, BD Pharmingen), anti-CD41 (PC7, P2 clone, Beckman Coulter), and anti-CD61 (FITC, SZ21 clone, Beckman Coulter). Antibodies were titrated to be well saturated. To evaluate platelet activation, samples were incubated with either 40 μM of thrombin receptor activating peptide 6 (TRAP) or PBS, together with the antibody mix. After incubation, samples were fixed for at least 10 min (1% paraformaldehyde, BD Cytofix), before dilution with PBS. Samples were kept at 4 °C in the dark and analyzed on BD LSR Fortessa within 48 h.

2.3. Functional effects of monoclonal anti-HPA-1a antibody measured by flow cytometry

Monoclonal anti-HPA-1a (26-4) [14] with concentrations 150 and 200 μg/mL was kindly donated by Mariana Eksteen and Tor Stuge, Immunology research group, Arctic University of Norway, Tromsø, Norway. Citrated whole blood was incubated 1:1 with 26-4 (final concentration 20 μg/mL) or Tyrode's buffer for 30 min at 37 °C. Samples were then diluted 1:25 with Tyrode's buffer and stained with anti-PAC-1 (FITC, BD Biosciences), anti-CD62P, and anti-CD42a, with simultaneous activation using TRAP 40 μM (high) or 10 μM (low).

2.4. Preparation of washed platelets for fibrinogen binding experiments

Platelet rich plasma (PRP) was separated from whole blood by centrifugation (200*g*, 15 min), and platelets were further concentrated by a second centrifugation step (400*g*, 15 min). Platelet concentrations were measured using Casy TT (Roche Diagnostics). The platelet concentrates were diluted with Tyrode's buffer to a concentration of 10^7/ mL.

2.5. Binding of fluorescent fibrinogen in the presence of monoclonal anti-HPA-1a antibody

The binding of fibrinogen to platelets may be evaluated using fibrinogen conjugated with fluorescent dye molecules [15,16]. Washed platelets were simultaneously incubated 1:1 with anti-HPA-1a/26-4 (final concentrations 18.75 μg/mL and 38.5 μg/mL) or Tyrode's buffer and Alexa-488 conjugated fibrinogen (Thermo Fischer Scientific, ref. no. F13191, final concentration 0.2 mg/mL) for 30 min in the dark at 37 °C. The platelets were subsequently stained for 15 min at dark RT with anti-CD42a and simultaneous activation using TRAP (40 μM), washed (200*g*, 15 min), and fixed as described above.

2.6. Platelet immunofluorescence test (PIFT)

To confirm the specific binding of anti-HPA-1a/26-4 to HPA-1a positive platelets as previously published [14], a flow cytometry-based platelet immunofluorescence test was performed [17]. Briefly, whole blood in EDTA collection tubes was centrifuged to create PRP, washed, and incubated with 26-4 (50 μg/mL) or Tyrode's buffer for 30 min at 37 °C, then washed again and stained with anti-CD42a and anti-human IgG Fc (PE, clone HP6017, Biolegend).

2.7. Flow cytometry analysis – quantification of glycoproteins

Platelets were identified by forward and side scatter properties, and expression of CD42a. Median fluorescence intensities (MFIs) were analyzed from single cells (FSC-H/FSC-A). For all samples, data from at least 10,000 platelets was collected at < 2000 events/s for optimal resolution. To ensure a reliable quantification of anti-CD61 FITC/HPA-1a, Quantum beads (catalog code 555pA, Bangs Laboratories, Inc.)

were prepared per manufacturer's instructions for each acquisition, and the accompanying software (QuickCal, version 2.3), allowed for standardization of the FITC MFIs into molecules of equivalent soluble fluorochrome (MESFs). To visualize stability of other fluorochromes, compensation beads (OneComp beads, eBioscience/Affymetrix) were prepared fresh according to the manufacturer's instructions and compared for each acquisition. In addition, quality controls were performed on the instrument daily (BD CSTs). Data was collected using BD FACSDiva software, and analyzed with FlowJo software (v.10.0.8rl for Mac OS X, FlowJo, LLC).

2.8. Flow cytometry analysis – experiments with monoclonal anti-HPA-1a antibody (26-4)

Data from the experiments on anti-HPA-1a (26-4) antibody effects on platelet activation (CD62P, PAC-1) and fibrinogen binding was acquired similarly as described above, but using the CytoFLEX flow cytometer (Beckmann Coulter) with the accompanying software (CytExpert).

2.9. Platelet aggregation tests – Multiplate and Rotem

To assess platelet aggregation in adults and neonates, samples were tested with impedance aggregometry in Multiplate (Roche Diagnostics), and with thromboelastometry in Rotem (Tem International GmbH). For Multiplate, 300 μL of citrated whole blood was incubated with 300 μL 0.9% NaCl solution with 3 mM $CaCl_2$ for 5 min before TRAP or ADP were added as stimuli. Aggregation was subsequently measured for 6 min as area under the curve (AUC) in arbitrary units (U). For adult and neonatal comparisons, Rotem tests were performed per manufacturer's instructions, with Extem (tissue factor) as stimulus. To measure the effect of anti-HPA-1a antibody addition in the Rotem platform, 20 μL of 26-4 (150 μg/mL) was added to 400 μL umbilical cord blood (final concentration 7.14 μg/mL), and incubated for 5 min at 37 °C before stimulation, with 20 μL Tyrode's buffer as control. Platelet aggregation, clot firmness and fibrinolysis were measured for 60 min.

2.10. Statistical analyses

Normality distributions were evaluated with Shapiro-Wilks test. For comparisons of continuous data, Student's *t*-test for independent samples or Mann-Whitney *U* test was used, depending on distribution of the data. ANOVA or Kruskal-Wallis tests were used for multiple comparisons. For correlations, Spearman's or Pearson's tests were used. A p-value of < 0.05 was considered significant. IBM SPSS Statistics for Mac, version 22 (IBM Corp., Armonk, N.Y., USA) was used for all analyses, while figures were created using GraphPad Prism 6 for Mac OS X, version 6.0d (GraphPad Software, La Jolla California, USA).

3. Results

3.1. Neonatal platelets are less responsive to TRAP stimulation compared to adult platelets

It has been suggested that neonatal platelets are less responsive to stimulation *in vitro* compared to adult platelets, which could potentially contribute to the pathogenesis of FNAIT. To further investigate and extend this notion, we measured the responsiveness of neonatal umbilical cord blood platelets to platelet activation in comparison with adult peripheral blood platelets using flow cytometry. Umbilical cord blood platelets had similar basal expression of CD62P and CD41, slightly lower expression of PAC-1, and higher expression of CD42a (previously shown to decrease with activation [18]) (Table 1). Activation-induced changes known to take place for these markers were less pronounced in cord blood compared to adult platelets after stimulation with the platelet agonist TRAP (Table 1, Fig. 1).

The platelet HPA-1a epitope expression was also quantified and compared between the groups, using an HPA-1a allele-specific monoclonal antibody directed against the GPIIIa chain (CD61) of the GPIb/IIIa glycoprotein [19,20]. Neonatal platelets had a tendency towards higher CD61/HPA-1a expression compared to adult platelets, but this difference was statistically significant only on resting HPA-1a homozygous platelets (Table 1). Both adult and neonatal HPA-1a homozygous platelets had a higher CD61/HPA-1a expression than platelets heterozygous for HPA-1a, which represents the fetal genotype in FNAIT. In all samples the CD61/HPA-1a expression increased with activation of platelets by TRAP, but as for the other markers, the neonatal change upon activation was on average lower compared to the adult. Overall, these data suggest a lower *in vitro* response to TRAP in neonatal platelets compared to adult platelets.

3.2. Umbilical cord blood platelets show reduced in vitro platelet aggregation

In line with less pronounced phenotypic changes after TRAP activation in flow cytometry, neonatal platelets also aggregated less efficiently *in vitro*. In Multiplate analysis, umbilical cord blood platelets showed a markedly reduced capacity to aggregate with both ADP and TRAP as stimuli (Fig. 2). In Rotem (Supplementary Fig. 1), the differences between adult and umbilical cord blood samples were marginal, but umbilical cord blood samples had a significantly shorter clotting time (CT), a tendency towards reduced maximum clot firmness (MCF) and longer clot formation time (CFT, Supplementary Fig. 1 and data not shown). A shorter clotting time indicates an enhanced primary hemostasis in neonatal blood, while the other parameters points to a reduced platelet function. Taken together, our data suggest a strongly reduced capacity of neonatal platelets to respond to agonists, and a less pronounced, yet apparent, diminished platelet function in more physiological aggregation and coagulation assays.

The cellular compositions of the first group of 12 adult and 12 cord blood samples were determined (Supplementary Table 1). Umbilical cord blood samples contained higher white blood cell counts and fewer, but larger, erythrocytes compared to adult peripheral blood, but importantly, the platelet counts were similar between the groups. Thus, the number of platelets could not explain reduced aggregation measured in cord blood. Cord blood samples had a slightly lower mean platelet volume (MPV) compared to adult platelets (Supplementary Table 1), but we did not find any correlation between MPV and aggregation in Multiplate induced by TRAP or ADP for either adult or cord blood platelets (Supplementary Fig. 2). There was also no correlation to CD62P up-regulation (data not shown). We cannot exclude that differences in WBC and RBC counts could influence the functional tests, but because functional deficiency was also observed on the single platelet level, we consider those differences unlikely to explain reduced platelet function in the assays. We conclude that the major differences in functional data between the groups are unlikely to be due to variations in platelet counts, platelet volume or indirectly by other cells. As such, our data confirm and extend the previous notion that umbilical cord blood platelets are hypo-responsive relative to platelets from adult peripheral blood.

3.3. Monoclonal anti-HPA-1a antibody reduces fibrinogen binding to adult and neonatal platelets

Fibrinogen binding to the open conformation of the GPIIb/IIIa glycoprotein is essential for platelet activation and aggregation, as subsequent cross-linking of activated platelets further enhances platelet activation and clot formation [12]. To test if maternal alloantibodies against the HPA-1a epitope on GPIIb/IIIa could interfere with fibrinogen binding on fetal/neonatal platelets, we asked if fibrinogen binding to washed platelets could be reduced by incubation with the monoclonal HPA-1a-specific alloantibody 26-4. We first verified that

Table 1

Platelet glycoproteins median fluorescence intensities (MFIs) in adult and umbilical cord blood. Table with mean (range). N = 12 for CD41, CD42a and CD62P. For CD61: MESF units. N = 9 for HPA-1aa adults, n = 3 for HPA-1ab adults, n = 8 for HPA-1aa cord blood, n = 4 for HPA-1ab cord blood. For PAC-1, the results are from a second set of experiments, where n = 6 for umbilical cord blood samples, and n = 10 for adult samples. Platelets were activated using TRAP (40 μM).

	Resting			Activated		
	Adult	Cord blood		Adult	Cord blood	
CD61, HPA-1aa	24,869 (17,860–35,736)	32,918 (22,663–41,640)	p = 0.015	34,194 (23,287–50,017)	41,203 (32,610–52,383)	NS
CD61, HPA-1ab	16,126 (11,215–18,773)	18,156 (16,165–21,997)	NS	22,668 (15,743–29,286)	23,916 (19,882–26,755)	NS
CD41	22,998 (16,691–31,793)	21,577 (17,613–26,321)	NS	30,738 (21,871–40,150)	25,143 (19,995–32,022)	p = 0.018
CD42a	5123 (3586–8171)	7242 (4643–8600)	p = 0.004	3492 (2143–6460)	6542 (3975–7992)	p < 0.001
CD62-P	68.6 (26–162)	91.5 (45–257)	NS	2292 (866–3145)	903.5 (233–2405)	p < 0.001
PAC-1	3326 (1663–6378)	1437 (947–2394)	p = 0.002	88,823 (18,950–168,634)	8199 (2837–17,592)	p < 0.001

Alexa-488-conjugated fibrinogen bound to adult and cord blood platelets, and found similar binding to resting platelets from these two sources (Supplementary Fig. 3). In addition, fibrinogen binding increased on both adult and umbilical cord blood platelets after activation (Supplementary Fig. 3), in line with our data on the increase in PAC-1 expression after TRAP stimulation (Fig. 1, Table 1). The increase seemed to be more pronounced on adult platelets, but the differences did not reach statistical significance. We also tested specific binding of 26-4 to HPA-1a-positive platelets with PIFT, and verified that it distinguished HPA-1aa, HPA-1ab and HPA-bb platelets as expected (Supplementary Fig. 4).

We then proceeded to test if 26-4 would interfere with the binding

of fibrinogen to platelets. We found that fibrinogen binding to platelets was reduced in the presence of 26-4 on HPA-1aa and HPA-1ab donors, but not to platelets from HPA-1bb donors (Fig. 3A and B). As expected, reduction was most pronounced on HPA-1aa platelets compared to HPA-1ab platelets. However, it was clear that the presence of 26-4 reduced fibrinogen binding also to platelets expressing the HPA-1a epitope on only half of the fibrinogen receptors (HPA-1ab cells), suggesting a possible biological effect of the anti-HPA-1a antibody also on heterozygous cells. Interestingly, TRAP activation did not override the effect of 26-4 inhibition of fibrinogen binding, suggesting a strong and dominating effect also in a situation of enhanced fibrinogen binding (Fig. 3B).
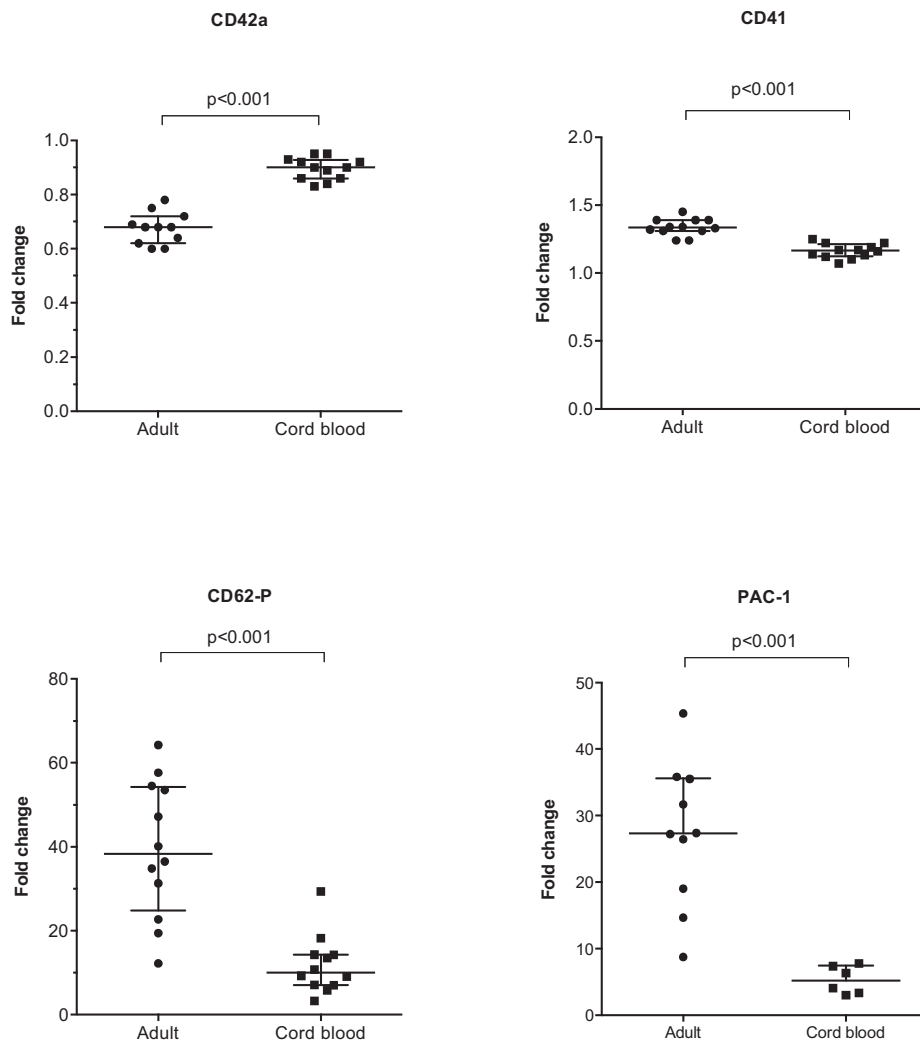

Fig. 1. Changes in platelet glycoprotein expression on adult peripheral and neonatal umbilical cord blood platelets following TRAP activation. Adult and cord blood samples were stained for CD42a, CD41, CD62P and PAC-1. Platelets were stimulated using TRAP (40 μM). Each dot represents one donor and depicts the relative change in marker expression after stimulation. The experiments comparing the increase of PAC-1 were performed on a different set of samples compared to the other three markers.
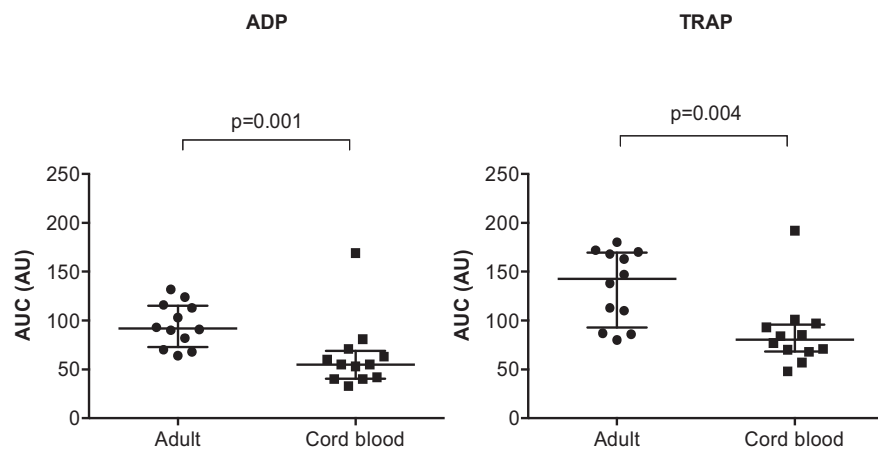
ADP

TRAP

Fig. 2. Adult and neonatal platelet aggregation measured in Multiplate. Citrated whole blood samples from adults and umbilical cord blood were stimulated with ADP or TRAP. The data is plotted with median and interquartile range. The outlying cord blood sample had a leukocyte count of 20.9×10^9/L, and a platelet count of 358×10^9/L, which both could have contributed to an increased aggregation.
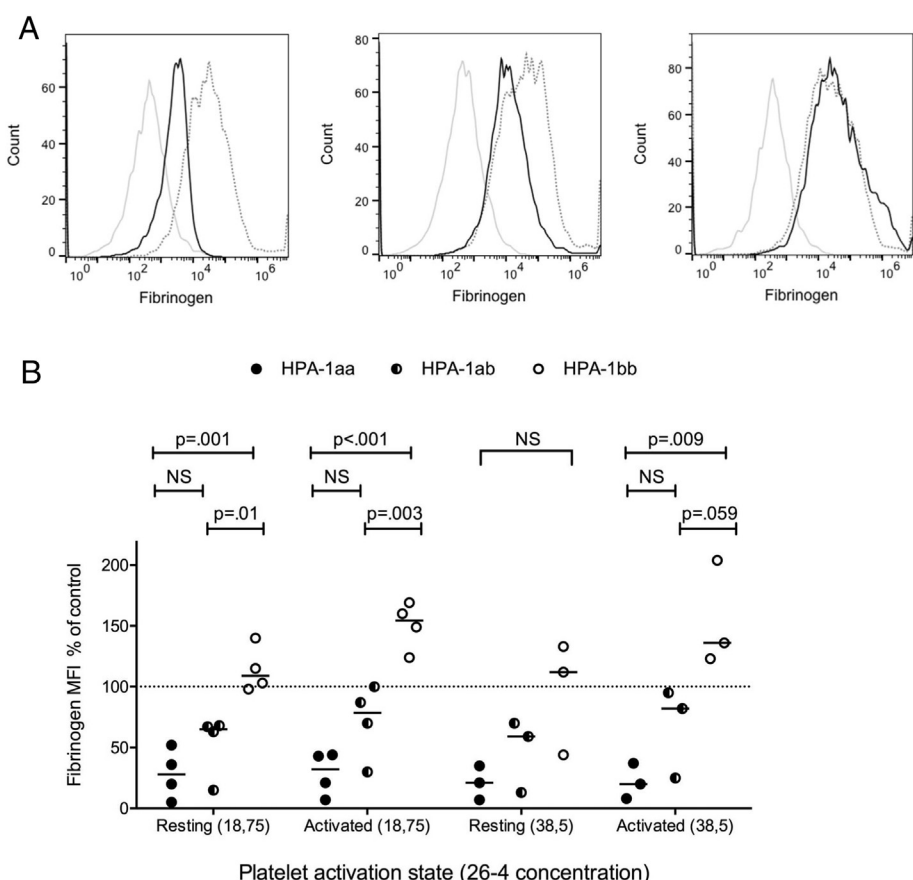

Fig. 3. A, B: Fibrinogen binding in the presence of anti-HPA-1a (26-4). A) Representative histograms from one experiment. From left to right: Activated HPA-1aa, 1ab, and 1bb samples. Black histogram = sample with 26-4, dotted histogram = control sample, light grey histogram = background (CD42b single stain). B) A summary of four experiments with 3–4 adult donors of each HPA-1 type, depicting fibrinogen binding in the presence of 26-4 as percent of fibrinogen binding in the absence of 26-4. p-Values are from Bonferroni post-hoc pairwise comparisons after ANOVA.

C: Effect of the anti-HPA-1a antibody 26-4 on CD62-P up-regulation. Adult peripheral whole blood was incubated with 26-4 or with control buffer, before staining and activation of platelets. Quantification of MFIs from four experiments with donors of different HPA-1a types (n = 4 for HPA-1aa and 1ab, n = 3 for HPA-1bb). Lines represent median and interquartile range.

D: Fibrinogen binding to umbilical cord blood platelets, similar experiment as in 3A and 3B. Filled circles represent HPA-1aa samples (n = 18), while half-filled circles represent HPA-1ab samples (n = 2).

We next set out to test if pre-incubation of whole blood with 26-4 would affect platelet activation by TRAP, an agonist for the thrombin receptor. We found a tendency towards reduced platelet activation after TRAP-stimulation in the presence of 26-4 in HPA-1aa and 1ab samples, but not for HPA-1bb samples, though none of these effects reached statistical significance (Fig. 3C). In the same sets of experiments, 26-4-incubation resulted in a clear reduction in binding of PAC-1, recognizing the activated form of the fibrinogen receptor, indirectly confirming blocking of the fibrinogen receptor by anti-HPA-1a (Supplementary Fig. 5).

Because targets of anti-HPA-1a antibodies in FNAIT are fetal or neonatal platelets, we investigated if 26-4 would also block fibrinogen binding to umbilical cord blood platelets. Similar to adult platelets, 26-4 reduced fibrinogen binding to HPA-1aa neonatal platelets, albeit to a
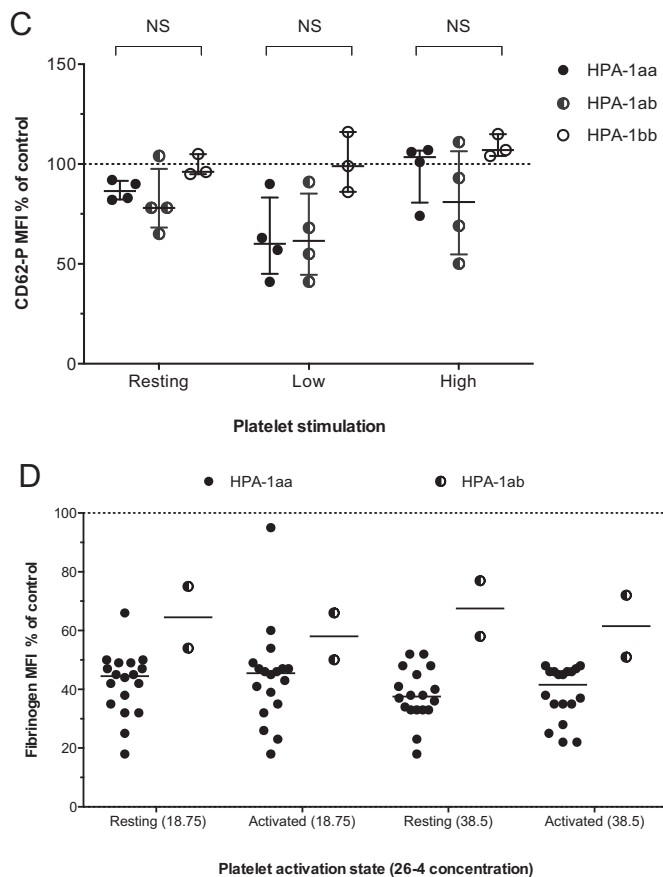
Fig. 3. (*continued*)

Fig. 4. Effects of 26-4 on Rotem parameters, relative to control. Umbilical cord blood samples, n = 4 for HPA-1aa and n = 3 for HPA-1ab. CT = clotting time, CFT = clot formation time, MCF = maximum clot firmness, Alpha = α-angle, reflecting the slope of tangent at 20 mm amplitude. CFT and α-angle reached statistical significance when comparing between HPA-1aa and HPA-1ab samples. For HPA-1aa samples, the α-angle (p = 0.003) and MCF (p = 0.011) were also significantly reduced when compared to control. For HPA-1ab samples, there were no statistical significant differences when comparing samples with and without antibody.

somewhat lesser extent than on adult platelets (Fig. 3D, filled circles). Because these samples could not be typed for HPA-1a in advance, we could not deliberately identify and select donors of the right genotype in this series of experiments. We were unlucky not to identify more than two HPA-1ab cord blood samples suitable for this analysis, but for both of those, we found that the 26-4 antibody inhibited fibrinogen binding (Fig. 3D, half-filled circles), thus supporting the data obtained using adult platelets (Fig. 3B). We conclude that the 26-4 antibody inhibits fibrinogen binding to adult and umbilical cord blood platelets alike, also to HPA-1ab heterozygous platelets, suggesting a potential clinically relevant effect *in vivo*.

3.4. 26-4 reduces clot formation in umbilical cord blood in Rotem analyses

Finally, we tested how the addition of 26-4 would affect platelet-dependent blood clotting in neonatal samples using the Rotem platform. A clear effect of 26-4 was seen on HPA-1aa samples, where a prolongation of the clot formation time (CFT) and a concomitant reduction of the alfa angle (slope of tangent at 20 mm amplitude), both reflecting reduced kinetics of the coagulation process, were seen (Fig. 4, Supplementary Fig. 6). In contrast, time to the initiation of the clot (CT) and the maximum clot firmness (MCF) were not significantly affected. Three HPA-1ab heterozygous samples could be included in this analysis; they were not affected by 26-4 in this analysis platform (Fig. 4, Supplementary Fig. 6).

4. Discussion

This study aimed to highlight the potential role of neonatal platelet function in FNAIT, and the effect of anti-HPA-1a antibodies on fibrinogen binding. Three major findings add complexity to what could determine the clinical outcome in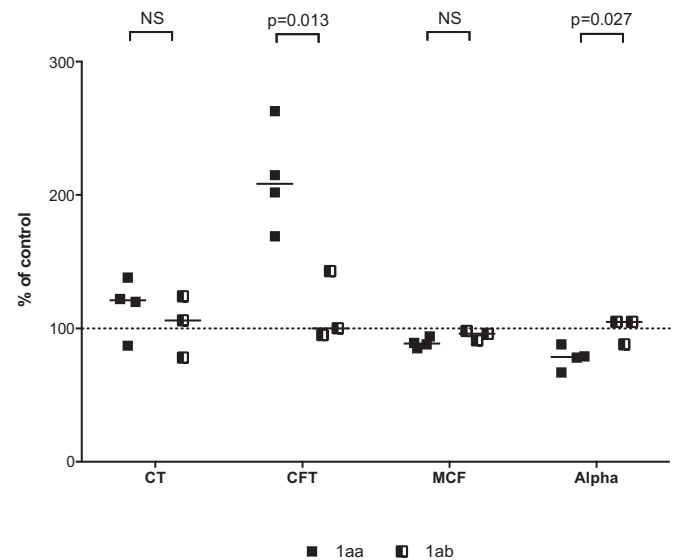 FNAIT. First, we confirmed and extended previous notions of reduced *in vitro* neonatal platelet function compared to adult platelets, which could render neonates more vulnerable to the effects of a low platelet count. Secondly, we found that the binding of anti-HPA-1a antibodies to the epitope on the fibrinogen receptor impaired fibrinogen binding, which could further decrease platelet function and disrupt the neonatal hemostatic balance. Lastly, platelet activation appeared to mildly increase expression of the HPA-1a epitope, which could enhance alloantibody binding and subsequently antibody-mediated effects such as platelet clearance.

Our findings on the expression of the activation markers CD62P and PAC-1 is similar to previous studies, showing comparable expression levels on resting platelets, but with a markedly higher expression on stimulated adult platelets compared to stimulated umbilical cord blood platelets [21,22]. In line with a previous study, we also found CD42a, part of the von Willebrand factor receptor complex, to be higher in our neonatal samples, which has been suggested to correspond with larger multimeric vWF in neonatal blood [23]. The explanation for why CD42a decreases with activation is controversial [18], but the fact that this down-regulation was less pronounced in umbilical cord blood platelets provided additional evidence for reduced responsiveness to TRAP. Apart from indicated lower responsiveness to TRAP activation, we also found a reduced surface expression of CD41 with activation. This was also shown in a previous study [22], and could, together with a reduced up-regulation of PAC-1 with activation in umbilical cord blood [24], suggest a decreased capacity for fibrinogen to activate platelets in neonatal hemostasis. Few studies have quantified HPA-1a expression directly using an HPA-1a allele-specific antibody clone [20]. Previous studies showed a slightly increased expression of CD61 in adults compared to neonates, disregarding HPA-1a type [21,22]. In our study, HPA-1a/CD61 expression was higher on HPA-1aa umbilical cord blood platelets, contrasting to those data. Furthermore, HPA-1a expression increased with activation of platelets, with up to 48% on cord blood heterozygous platelets, suggesting that, like the expression of CD41 and CD61 as such, HPA-1a epitope expression is dynamic. CD61 associates with CD41 (GPIIb, integrin α_{IIb}) to form the fibrinogen receptor (GPIIb/IIIa, integrin $\alpha_{IIb}\beta_3$), and it was therefore surprising that HPA-1a showed a higher basal expression while CD41 did not. One

possible explanation for this discrepancy may be an independent expression pattern of CD41 and CD61/HPA-1a in some instances. Alternatively, our results may be caused by technical issues related to the reagents used.

Similar to previous studies [25,26], we found that both ADP- and TRAP-stimulated umbilical cord blood platelets aggregated less well compared to adult platelets. Reduced expression of the fibrinogen receptor and less degranulation with activation of platelets is consistent with this finding. In Rotem, the finding of a shorter CT, and reduced CFT and MCF, is in line with the biggest study measuring platelet aggregation initiated by Extem/tissue factor so far [27]. The less pronounced differences between adults and umbilical cord blood in Rotem, despite the higher dependency on fibrinogen for this assay, may shed light on compensatory mechanisms for a reduced platelet function in neonates, such as increased levels of large vWF multimers [28], and reduced anti-thrombin and tissue factor pathway inhibitor [29]. This notion is supported by studies demonstrating shorter closure times with the PFA-100 for cord blood platelets [30], which seems dependent on factors in plasma [31].

A main aim of this study was to investigate if anti-HPA-1a antibodies could interfere with GPIIb/IIIa function. It has previously been hypothesized that anti-HPA-1a antibodies could block fibrinogen binding on the GPIIb/IIIa glycoprotein. Our study is to our knowledge the first to show such an effect directly on adult and umbilical cord blood platelets, not relying on transfected cell lines or on solid surface coated with fibrinogen [32,33]. Of note, the monoclonal anti-HPA-1a antibody used in our study was derived from an immunized mother who had given birth to two children with severe thrombocytopenia and skin bleedings [14]. This, and the fact that the antibody clearly blocked fibrinogen binding also to HPA-1ab heterozygous platelets, suggests a direct clinical relevance to our results.

The HPA-1a epitope is exposed on the surface of the GPIIIa subunit on both resting and activated platelets, and is distal to the fibrinogen binding site [34]. Though not directly investigated here, this suggests that the inhibition of fibrinogen binding is a form of steric hindrance. This may be relevant to the physiological role of fibrinogen *in vivo*, which is, in addition to being a precursor to fibrin, to facilitate clot formation by cross-linking platelets that have been activated by various agonists [35]. A form of steric hindrance, rather than a complete blocking of the fibrinogen-binding site, is supported by previous data suggesting only a moderate reduction in aggregation in Multiplate after addition of 26-4 for HPA-1a positive platelets [14]. In this paper, we were able to show a blocking effect by 26-4 on clot formation in umbilical cord blood samples, and thus identified a clinical effect of 26-4 in this physiological setting. In particular, we noted a prolonged clot formation time and a reduction in alpha-angle, both reflecting platelet function and fibrinogen levels. Of note, HPA-1ab samples were not inhibited by 26-4, suggesting that the number of GPIIb/IIIa molecules of the HPA-1b allotype in the heterozygous samples is sufficient to convey full effect of the platelets. This is perhaps not unexpected, since Rotem is an optimized coagulation platform with an unrestricted availability of pro-coagulation factors. In addition, the amount of blocking antibody was rather low in our experiments. Further quantitative studies on how anti-HPA-1a antibodies could interfere with fibrinogen binding and affect platelet activation and clot formation are needed.

A limitation of our study is the use of umbilical cord blood instead of neonatal peripheral blood, which was due to the large amounts of blood required for the functional assays. We also only included term newborns in our study, and previous studies have shown a further decreased platelet function in preterm babies [27,36]. This could be relevant to FNAIT, as severe bleedings may occur early in gestation [13]. In addition, our study was limited to the use of TRAP, ADP and tissue factor as platelet stimuli, but other activators could have revealed other differences. We also used a single monoclonal anti-HPA-1a antibody in our assay system. During pregnancy, the fetus will be exposed to a number

of different clones of anti-HPA-1a with different binding affinities, and it is possible that our monoclonal antibody represents a unique case. In a previous study, only 2 of 43 maternal sera specifically inhibited fibrinogen binding to HPA-1a transfected cells [33], indicating that functional blocking of fibrinogen binding may indeed represent rare situations. Moreover, it may be difficult to translate functional effects on purified proteins *in vitro* to clinical effects *in vivo*. Further studies on neonatal platelets, in a similar way as we have done here, are needed on a larger number of maternal anti-HPA-1a antibodies to investigate this question in more detail.

In conclusion, several mechanisms may contribute to the severity of FNAIT, which is reflected by the variability in clinical outcome. Our study confirms that anti-HPA-1a antibodies interfere with fibrinogen binding, which potentially could add to reduced neonatal platelet function in determining the severity of bleedings in FNAIT and act.

Conflicts of interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2017.12.017.

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