ORIGINAL ARTICLE

Reduced platelet function in preterm neonates compared with term neonates

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Abstract

Background: A reduced platelet function might contribute to the longer bleeding time seen in preterm neonates. However, the previously used platelet function testing in neonates is limited due to methodological limitations, mainly caused by difficulties in obtaining adequate blood volume. Therefore, the platelet function in preterm neonates is sparsely investigated. The aim of this study was to compare platelet function in preterm neonates at birth and at expected term age with platelet function in term neonates at birth.

Methods: We included 43 preterm neonates born at gestational age (GA) 28 + 0 to 34 + 0 and 21 term neonates born at GA 38 + 0 to 41 + 0. Within the first 24 hours of life, 1–1.5 mL peripheral blood was obtained and for preterm neonates, resampling was performed at expected term age (GA 38 + 0 to 41 + 0). Platelet function testing included impedance aggregometry and platelet activation measured by flow cytometry. In addition, platelet count was determined.

Results: Platelet count and platelet activation were reduced in preterm neonates compared with term neonates at birth, but we found no difference in impedance aggregometry at birth. At expected term age, platelet count and aggregation exceeded term levels, but platelet activation remained impaired in the preterm.

Conclusion: Preterm neonatal function is decreased at birth and does not seem to reach term levels during the first 4 to 13 weeks of life.

KEYWORDS
flow cytometry, infant, premature, platelet activation, platelet count, platelet function tests

Essentials

- Reduced platelet function might contribute to the longer bleeding time seen in preterm neonates.
- Previous studies in preterm neonatal platelet function weeks after birth show variable results.
- Platelet count in preterm neonates exceeds term levels 7 weeks after birth.
- Platelet activation in preterm neonates remains reduced 7 weeks after birth.

1 | INTRODUCTION

Preterm birth, defined as delivery before gestational age (GA) of 37 weeks, has a worldwide prevalence of 15 million neonates annually, which is equivalent to 11% of all newborns.[1] During recent decades, the mortality of the youngest and smallest preterm neonates has improved by roughly 2% points.[2–4] This has led to an increasing number of neonates with severe morbidities and complications to prematurity.[2–4] Intraventricular hemorrhage (IVH) is a significant complication to extreme prematurity, being inversely correlated to GA.[5–7] The etiology of IVH is incompletely understood, but it is believed to be multifactorial.[8–11] Thrombocytopenia affects up to 70% of preterm neonates with birthweight <1000 g,[12] and studies have found that IVH is often co-observed with thrombocytopenia.[13,14] Nevertheless, platelet count alone appears to be a poor predictor for IVH.[14–17] Interestingly, it has been suggested that once the bleeding has occurred, a reduced platelet function in the preterm might contribute to expansion of the bleed.[18,19] However, as reviewed by our group, platelet function in preterm neonates is only sparsely investigated[13,20] and its potential contribution to morbidity remains unclear.

Examination of platelet function in neonates is challenged by the difficulty of obtaining adequate blood volume to assess platelet function. Therefore, the majority of previous studies have been conducted using umbilical cord blood rather than peripheral blood.[21–26] However, previous studies indicate that platelet function determined in umbilical cord blood does not reflect that of peripheral blood[27–29]; thus, interpretation of platelet function testing in umbilical cord blood should be performed with caution.

Previous studies investigating platelet function in peripheral blood suggest a reduced platelet function in both term and preterm neonates when compared with adults.[24,25,30–34] Nevertheless, it seems more relevant to compare platelet function in preterm neonates with term levels to explore the greater bleeding risk associated with prematurity.[35] Few studies, using different methods, have investigated preterm neonatal platelet function in peripheral blood compared with term neonatal platelet function.[29,36–42] The majority of these found platelet hyporeactivity,[29,37–40] reduced adhesion,[42] reduced aggregation,[41] and decreased levels of surface glycoproteins[29] in the preterm platelets. In contrast, Cowman et al.[36] found no difference in either adhesion or glycoprotein expression in preterm versus term neonatal platelets, and Linder et al.[42] found no difference in platelet aggregation. Thus, previous studies investigating preterm neonatal platelet function compared with term neonatal platelets are few, and the results are inconsistent or even contradicting.

The aim of this study was to investigate platelet count and platelet function in peripheral blood in healthy preterm neonates at birth and at expected term age. Further, we aimed to compare the platelet function in preterm neonates at birth and when they reach term age with platelet function in term neonates. We hypothesized that platelet function is reduced in preterm neonates at birth compared with term neonates.

2 | METHODS

2.1 | Study population

Preterm neonates born at GA 28 + 0 to 34 + 0 w and term neonates born at GA 38 + 0 to 41 + 0 weeks were included at the neonatal intensive care unit, Department of Pediatrics and Adolescent Medicine and Department of Obstetrics and Gynecology at Aarhus University Hospital, Denmark, between February 2018 and January 2021. A subset (n = 21 term neonates) of these neonates was included and published in a previous study in which the aim was to investigate platelet function in umbilical cord blood compared with peripheral blood.[27]

Preterm neonates were excluded if they had a birth weight <1000 g and term neonates if <3000 g. Neonates were excluded if they had morbidity within the first 24 hours of life with known effect on coagulation, congenital malformations, or history of hereditary platelet dysfunction among first-degree relatives; were monozygotic twins; or there was maternal preeclampsia or maternal intake of aspirin 4 weeks before labor. Neonatal clinical data were extracted from medical records and maternal clinical data were obtained through systematics interviews.

The study complied with the Declaration of Helsinki and was approved by the Danish Data Protection Agency (Ref. no. 1-16-02-30-18) and the Central Denmark Region Committees on Health Research Ethics (Ref. no. M-2018-13-18). Written informed consent was obtained from both parents before inclusion.

2.2 | Blood sampling and clinical data

A blood sample from the antecubital vein was obtained within the first 24 hours of life, and for the preterm neonates, resampling was done when reaching expected term age defined as GA 38 to 41 weeks. All samples were drawn with minimal stasis using a 21-gauge needle to ensure minimal platelet activation.[43] For platelet count analyses, 0.5 mL of blood was collected into EDTA tubes (Becton Dickinson Bioscience, San Jose, CA, USA), and 1.0 mL of blood was sampled into 3.2% sodium citrate tubes (Becton Dickinson Bioscience) for impedance aggregometry and flow cytometry analyses.

2.3 | Laboratory analysis

Platelet count was measured using a hematology analyzer (XN-9000, Sysmex Corp., Kobe, Japan).

Platelet aggregation was examined by whole blood impedance aggregometry employing ROTEM platelet (rotational thromboelastometry with a platelet module added) using adp-tem (final concentration 10 μM adenosine diphosphate [ADP]) and trap-tem (final concentration 36 μM thrombin receptor activating peptide 6 [TRAP]) (Tem-group, Munich, Germany) as agonists. Within 2 hours of blood sampling, 150 μL of citrated whole blood was

diluted 1:1 with 150 μL of preheated (37°C) ROTEM platelet diluent and incubated for 3 minutes followed by addition of 12 μL adp-tem or trap-tem. Impedance between the two wires was measured continually for 6 minutes after addition of an agonist. Platelet aggregation was quantified as area under the aggregation curve (AUC) in ohms/min.

Expression of platelet surface glycoprotein (GP) receptors and activation was analyzed by whole blood flow cytometry using a NAVIOS flow cytometer (Beckman Coulter, Miami, FL, USA) and processed in NAVIOS Software version 1.3. The flow cytometer was calibrated daily according to protocol by Rubak et al.[44] Platelet surface GP receptor and platelet activation analysis was initiated after 30 minutes and 1 hour of whole blood resting, respectively, and completed within 2 hours of blood sampling, as previously described by Rubak et al.[44] Five microliters of citrated whole blood were added to 60 μL of HEPES buffer diluted antibodies, followed by room-temperature incubation in the dark for platelet surface GP receptor analysis (15 minutes) and platelet activation (10 minutes). After incubation, the sample was fixated using 2 mL of fixation buffer (0.2% paraformaldehyde in phosphate-buffered saline). The following fluorescence-labeled antibodies were used for platelet surface GP receptor analysis: CD41a (GPIIb) Phycoerythrin-Cyanine 7 (PCy7) (Clone HIP8), CD42a (GPIX) eFluor450 (Clone GR-P), CD42b (GPIb) Phycoerythrin (PE) (Clone HIP1), CD49b (GPIa) Allophycocyanin (APC) (Clone P1H5), CD61 (GPIIIa) fluorescein isothiocyanate (FITC) (Clone VI-PL2), all purchased from eBioscience (San Diego, CA, USA) except CD42b-PE purchased from AH Diagnostics (Tilst, Denmark). The following fluorescence-labeled antibodies were used for platelet activation analysis: CD42b-PE (Clone HIP1) (AH Diagnostics); CD62p-APC (P-selectin, Clone P-sel.KO2.3) (eBioscience, San Diego, CA, USA); CD63-PECy7 (Clone H5c6) (Beckton Dickinson Bioscience) and anti-fibrinogen–FITC (polyclonal chicken) (Diapensia HB, Linköbing, Sweden). The following agonists were used for platelet activation (final concentrations): collagen-related peptide (CRP, 0.12 μg/ml) (University of Cambridge, UK); ADP (10.8 μM) (Sigma-Aldrich, St. Louis, MO, USA); TRAP (28.5 μM) (JPT, Berlin, Germany) and arachidonic acid (ARA) (0.58 mM) (Sigma-Aldrich). Platelets were identified through scatter and CD42b-PE positivity followed by exclusion of platelet-leucocyte and platelet-platelet aggregates within a forward scatter intensity and forward scatter peak plot. Expression of platelet surface GP receptors and activation dependent surface markers was determined on the basis of 5000 and 10 000 counts, respectively. Results are expressed by percentage of positive platelet (% positive platelets) and median fluorescence intensity (MFI) of the positive platelets. Gates for the expression of platelet surface GP receptors were set using matched negative isotype controls (eBioscience, San Diego, CA, USA). Gates in the negative control for expression of platelet surface GP receptors were set in a histogram as close to the negative population as possible. Gates in the negative control for expression of activation-dependent surface marker were set to include

1.5% to 2.0% positive platelets for bound fibrinogen and CD63 and 0.1% to 0.2% for P-selectin. Preanalytical activation was assessed by expression of P-selectin in resting platelets within 2 hours of blood sample collection.

In the present study, the term *platelet function* includes both platelet aggregation as measured by impedance aggregometry and platelet activation as measured by flow cytometry.

2.4 | Statistics

A formal sample size calculation was not possible due to lack of published data on impedance aggregometry and flow cytometric platelet activation in preterm neonates. Twenty term neonates and 40 preterm neonates were estimated to be sufficient to detect a relevant difference between groups.

Distribution of continuous variables was assessed through QQ plots. Normally distributed data are presented as mean ± standard deviation and tested with parametric tests; data not normally distributed are presented as median with interquartile range (IQR) or range and tested with nonparametric tests. In the figures, all data are presented as median with IQR. Differences between preterm neonates at birth and at expected term age were analyzed using a paired *t* test or Wilcoxon signed-rank as appropriate. Differences between preterm and term neonates were tested using an unpaired *t* test or Mann-Whitney *U* test. For tests of platelet count and platelet aggregation, the alpha level was set to 0.05. To adjust for multiple testing a Bonferroni correction was used on results obtained by flow cytometry comparing preterm neonates and term neonates; the corrected significance level for tests of GP receptor expression was 0.003 (0.05/15) and for tests of platelet activation 0.004 (0.05/12). Statistical analysis was performed in Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA).

3 | RESULTS

We included 43 preterm neonates and 21 term neonates who had a 7.4-week median difference in GA at birth and a 1632 g median difference in birth weight. The two groups were comparable with respect to sex but differed in delivery mode. Thirty-six (84%) preterm neonates suffered from respiratory distress syndrome (RDS), but none of the mature neonates did. None of the neonates had sepsis or any serious illness (see Table 1). We tested whether delivery mode, maternal smoking, or fish oil intake during pregnancy; maternal antenatal steroids; or sepsis or RDS among preterm neonates influenced the results, but we found no differences in any parameter between groups (data not shown).

Thirteen preterm neonates were lost to follow-up due to failed blood sampling (n = 2), clotted blood samples (n = 4), or parents withdrawing from the project (n = 7). At follow-up, the preterm neonates had a median corrected age of 39 + 3 weeks (range, 38 + 1–41 + 0)

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TABLE 1 Demographics and clinical characteristics of term and preterm neonates at delivery and maternal characteristics during pregnancy

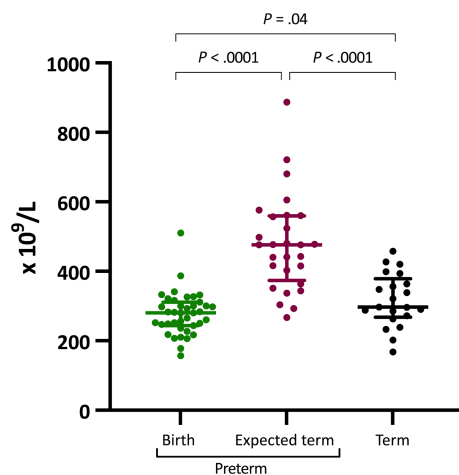| Neonatal data	Preterm neonates at birth (n = 43)	Term neonates at birth (n = 21)
Gestational age at delivery, wk, median (range)	32 + 4 (28 + 4–34 + 0)	40 + 0 (38 + 2–41 + 0)
Birth weight, g, median (range)	1950 (1050-2470)	3582 (3018-4605)
Male sex/Female sex	23/20	8/13
Caucasian race, n	43	21
Mode of delivery, n (%)		
Vaginal	24 (56)	18 (86)
Cesarean section	19 (44)	3 (14)
Apgar score at 1 min, n (%)		
7-10	36 (84)	20 (95)
4-6	6 (14)	1 (5)
1-3	1 (2)	0
Platelet count at birth, median (IQR)	280×10^9/L (244-311)	297×10^9/L (269-379)
Maternal data	**Giving birth to preterm (n = 41)**	**Giving birth to term (n = 21)**
Morbidity during pregnancy, n (%)		
Preeclampsia	3 (7)	0
Gestational diabetes	2 (5)	0
Gestational hypertension	3 (7)	1 (5)
Maternal fish oil intake during pregnancy, n (%)	13 (30)	1 (5)
Smoking during pregnancy, n (%)	3 (7)	0
Antenatale steroids, n (%)	34 (79)	0

Abbreviation: IQR, interquartile range.

FIGURE 1 Platelet count in 39 preterm at birth, 28 preterm at expected term age, and 21 term neonates at birth. Please, note that birth versus expected term is paired data and the remaining is unpaired data. *Note*: The bars indicate median and interquartile range

and a median weight of 3435 g (range, 2310–4160), which did not differ from the birth weight of the term neonates ($P = .1$). The median time between the blood sample at birth and at expected term age was 7.1 weeks (IQR, 6.2-8.3 weeks).

The following results are presented as median with IQR and tested with nonparametric tests, as data were not normally distributed. Differences between preterm neonates at birth and at expected term age were analyzed using a paired *t* test or Wilcoxon signed-rank as appropriate. Differences between preterm and term neonates were tested using an unpaired *t* test or Mann-Whitney *U* test.

3.1 | Platelet count

As shown in Figure 1, preterm neonates had a significant lower median platelet count than term neonates at birth ($P = .04$). When preterm neonates reached expected term age, the median platelet count was 476×10^9/L (IQR, $374–560 \times 10^9$/L), which was significantly higher than in both preterm and term neonates at birth.

3.2 | Platelet aggregation

As shown in Figure 2, no difference was found in platelet aggregation between preterm and term neonates at birth after stimulation with ADP and TRAP. When preterm neonates reached expected term age, we found a significantly higher platelet aggregation than in both preterm and term neonates at birth.

FIGURE 2 Platelet impedance aggregometry in 41 preterm at birth, 28 preterm at expected term age, and 21 term neonates at birth. Please note that birth versus expected term is paired data and the remaining is unpaired data. *Note*: The bars indicate median and interquartile range. ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide-6
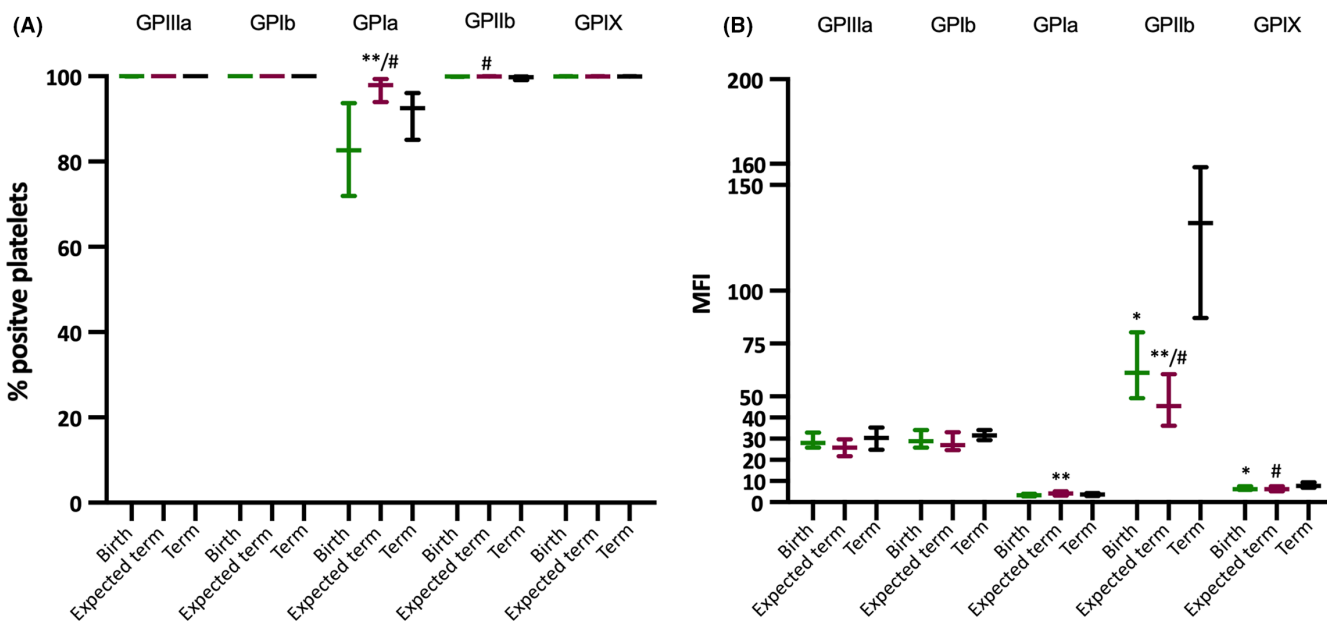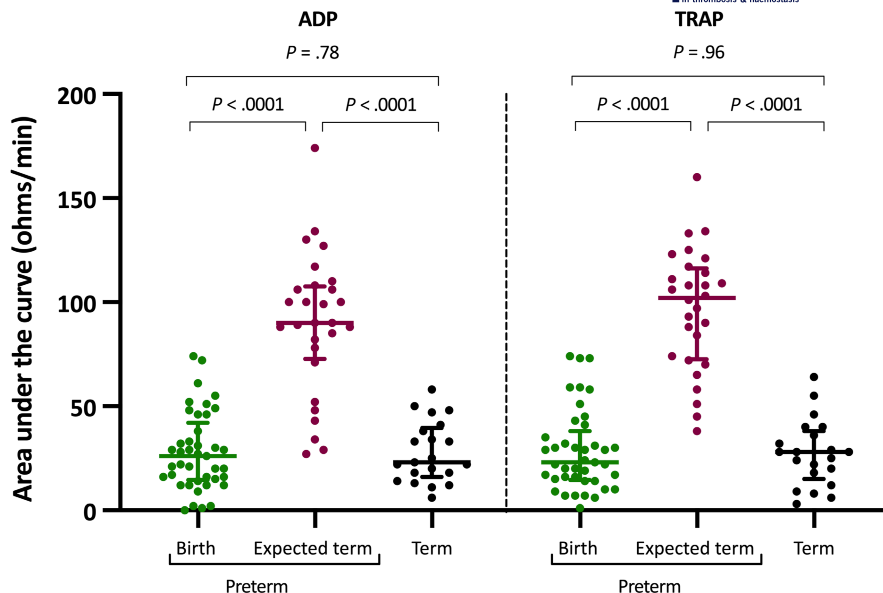
FIGURE 3 Expression of platelet surface glycoprotein receptors as (A) percentage of positive platelets and (B) platelet median fluorescence intensity in 41 preterm at birth, 30 preterm at expected term age, and 21 term neonates at birth. *Note*: The bars indicate median and interquartile range. Bonferroni corrected significance level: $P = .003$. Please note that birth versus expected term is paired data and the remaining is unpaired data. GP, glycoprotein; MFI, median fluorescence intensity

* Significantly different from term neonates
** Significantly different from birth levels
# Significantly different from term levels

3.3 | Platelet glycoprotein surface markers

Figure 3A shows the percentage of positive platelets and 3B shows platelet MFI (density) of platelet surface GP receptors expression (IIIa, Ib, Ia, IIb and IX) in which a Bonferroni adjusted significance level of 0.003 was applied. Compared with term levels, preterm neonatal platelets did not differ significantly in percentage of positive

platelets for any of the GP surface receptors, but we found significantly lower MFI of GPIIb and GPIX in the preterm. At expected term age, preterm neonatal platelets showed a significantly higher percentage of positive platelets and higher MFI of GPIa and lower MFI of GPIIb than birth levels. Compared with term neonates, preterm neonates at expected term age showed a significantly higher percentage of positive platelets for GPIa and GPIIb but lower MFI

of GPIIb and GPIX. GPIIIa and GPIb did not differ in percentage of positive platelets or MFI between groups.

3.4 | Platelet activation

We evaluated platelet activation expressed by bound fibrinogen, CD63, and P-selectin expression after addition of CRP, ADP, TRAP, and ARA. Results are shown as percentage of positive platelets in Figure 4 and as density of the activation markers (platelet MFI) in Figure 5, in which a Bonferroni adjusted significance level of 0.004 was applied. An overview of the results is provided in Table 2. No difference was found in preanalytical activation in the blood samples from preterm and term neonates (Table 3).

Preterm neonates showed significantly less percentage of positive platelets for bound fibrinogen, CD63, and P-selectin after addition of CRP compared with term neonates at birth. No differences in percentage of positive platelets were demonstrated between preterm and term neonates at birth after addition of ADP, TRAP, and ARA except for P-selectin after addition of ADP which was significantly different from term levels.

When preterm neonates reached expected term age, significantly more platelets were positive for bound fibrinogen, CD63, and P-selectin after addition of CRP, ADP, and TRAP compared with birth levels (all P values ≤ .003 except percentage of positive platelets for bound fibrinogen after addition of ADP, which did not reach statistical significance, $P = .01$). No difference was found in percentage of positive platelets between birth and expected term age in any activation-dependent markers after addition of ARA.

Preterm neonates at expected term age showed significantly more percentage of positive platelets for bound fibrinogen and CD63 after addition of ARA and for P-selectin after addition of TRAP than term neonates at birth.

Preterm neonates showed significantly lower MFI for bound fibrinogen after addition of CRP, CD63 after addition of TRAP, and P-selectin after addition of CRP, ADP, and TRAP than term neonates at birth.

At expected term age, preterm neonates showed significantly lower MFI for bound fibrinogen after addition of ADP than their levels at birth, and for P-selectin we found significantly higher MFI after addition of CRP and TRAP at expected term age than at birth levels.

Preterm neonates at expected term age showed significantly lower MFI for bound fibrinogen, CD63, and P-selectin after addition of CRP and ADP than term neonates. For bound fibrinogen after addition of ARA the MFI was significantly higher in preterm neonates at expected term age compared with term neonates.

4 | DISCUSSION

The main findings of this explorative study are that when reaching expected term age, preterm neonates had increased platelet counts, increased platelet aggregation, and increased platelet activation markers compared with their birth levels. Interestingly, and a novel finding, platelet activation markers did not reach the levels of term neonates at expected term. For most GP surface receptors, no difference was found between preterm and term neonates, but, remarkably, GPIIb and GPIX receptors remained reduced in preterm neonates 7.1 weeks after birth.

The preterm neonates investigated in this study had lower platelet counts than term neonates at birth, though this was clinically insignificant. Supporting our results, Wiedmeier et al,[45] who investigated 47 291 neonates born at GA 22 to 42 weeks found increasing platelet count with advancing GA at birth and increasing count with advancing postnatal age. They showed increased postnatal platelet count at two peaks seen 2 to 3 weeks (mean count, 450 000) and 6 to 7 weeks postnatally (mean count, 510 000), and this variation could potentially explain the rather broad variation found in the expected term neonates in this study.

Previous studies investigating platelet aggregation reported no difference between preterm and term neonates at birth.[41,42] One study demonstrated a postnatal increase during the first 10 to 14 days of life.[41] In our study, we found an increase 4 to 13 weeks after birth, which could indicate that preterm platelet aggregating is increased several weeks postnatally. Of note, a positive correlation between impedance aggregometry and platelet count is demonstrated in studies in adults using the multiplate analyzer, which employs impedance aggregometry like the ROTEM platelet does.[46–48] Presuming this correlation also exists in neonates and when using ROTEM platelet, this would explain the postnatal increase in aggregation found in our study.

Only few previous studies investigated platelet GP expression in preterm neonates compared with term in peripheral blood, and the results are contradicting.[29,36,37] Koltsova et al.[37] did not find any difference in GPIb surface expression when comparing preterm neonates born with GA 32 to 34 weeks and term neonates, which is in line with our findings. Conversely, Cowman et al.[36] found increased levels of GPIb in neonates born with GA <32 weeks. Sitaru et al.[29] reported no difference in any GP surface receptors between preterm and term neonates except significantly lower levels of GPIIIa in preterms <30 weeks. None of these studies can support our finding of lower density (MFI) of GPIIb and GPIX in preterm platelets than terms at birth, and to the best of our knowledge this finding is new. However, the numerical results for GPIX are very close, regardless of the statistical significance, and we do not expect this to have any clinical impact. Whether the reduced GPIIb receptors has implications for the in vivo coagulation is difficult to conclude yet, and larger studies must be conducted to further clarify this.

Reduced levels of activation dependent markers in neonates compared with adults are well established,[29–32,34,49,50] but term neonates display no bleeding tendency like preterm neonates do.[35] Therefore, when investigating preterm neonatal platelet function, it seems most accurate to compare with term neonates as we did in this study. Our finding of overall reduced levels of activation-dependent
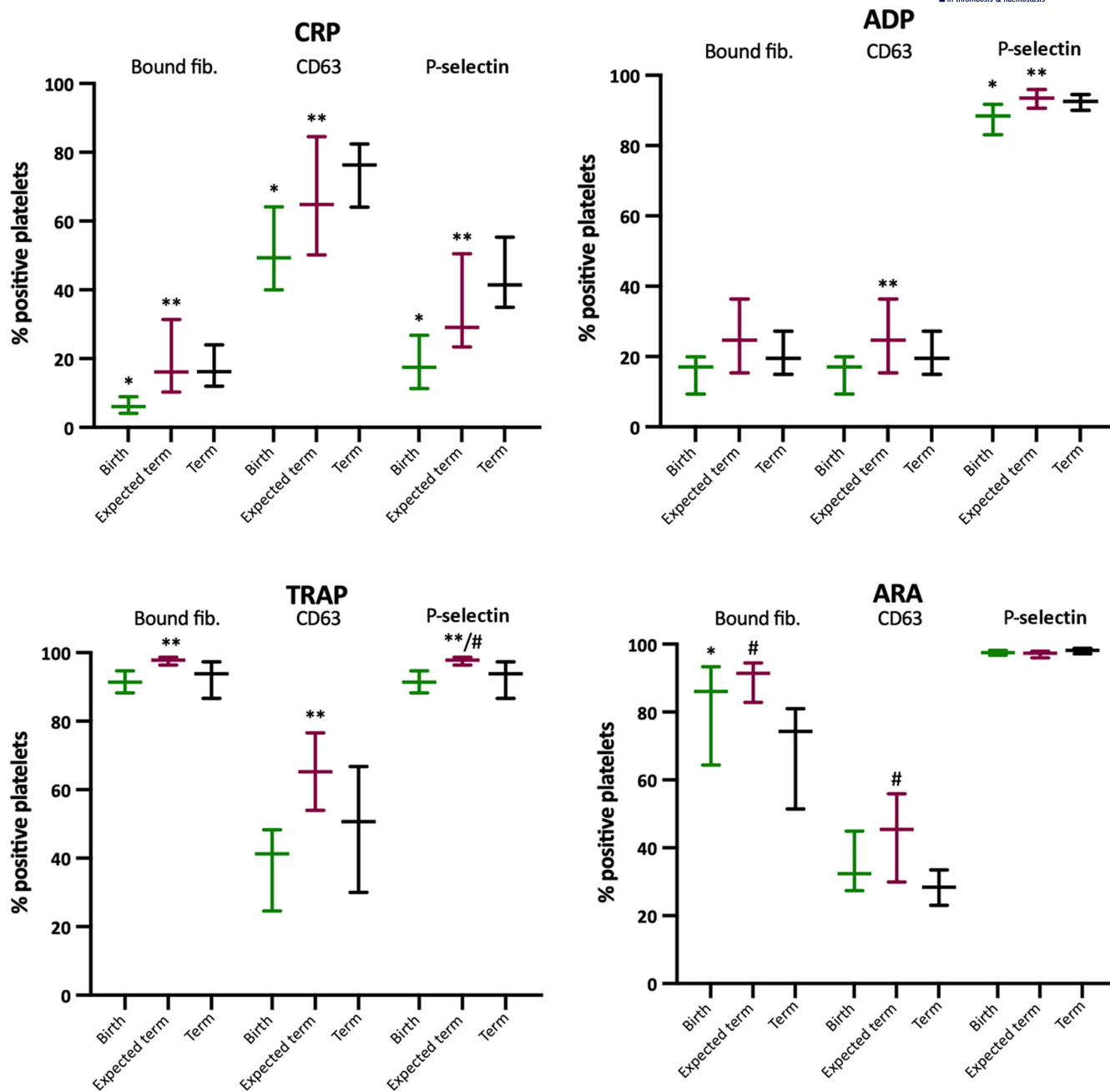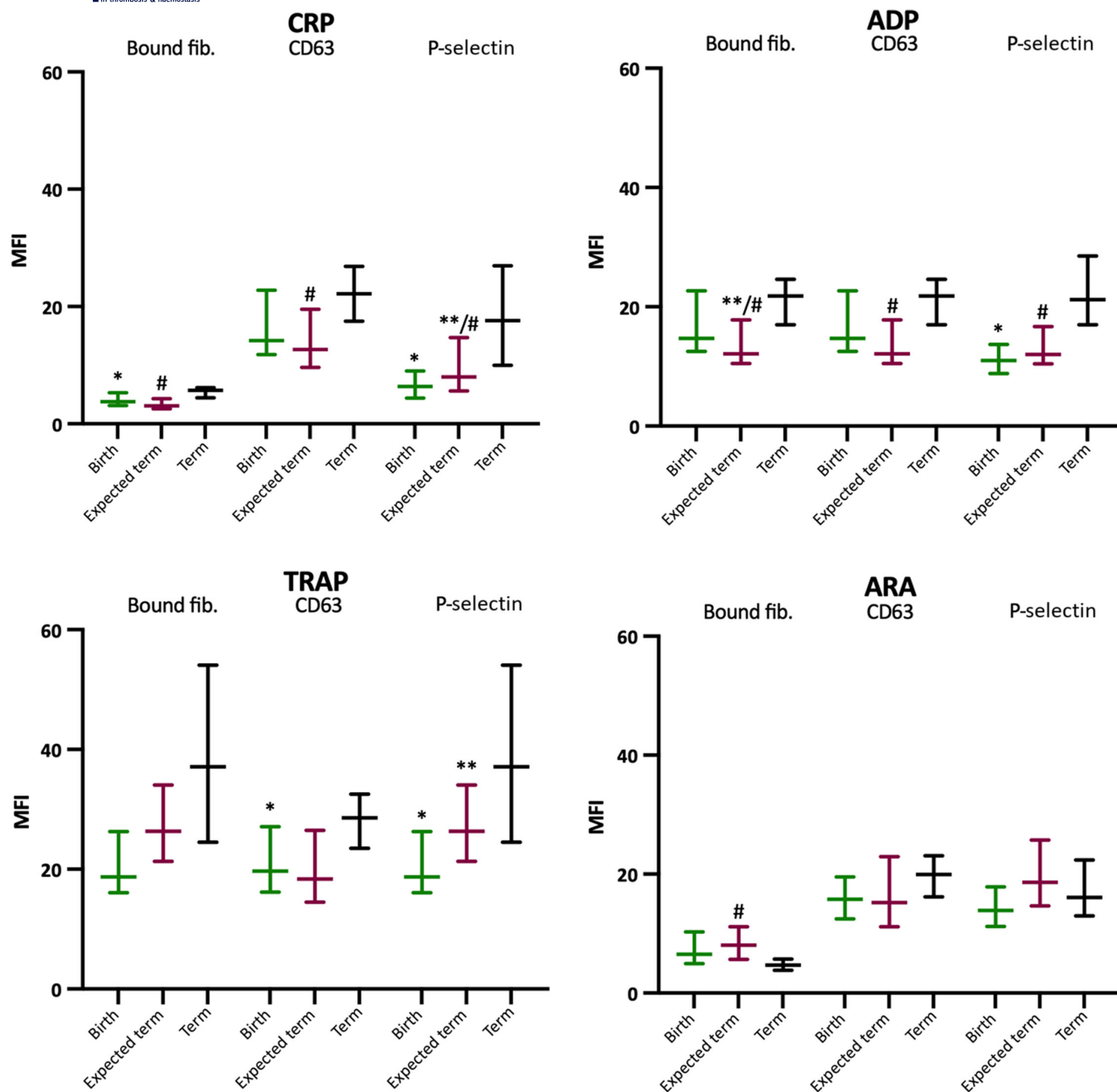
* Significantly different from term neonates
** Significantly different from birth levels
# Significantly different from term levels

FIGURE 4 Percentage of platelets expressing activation-dependent surface markers–bound fibrinogen, CD63, and P-selectin when activated by agonists collagen-related peptide, adenosine diphosphate, thrombin receptor-activating peptide 6 and arachidonic acid in 43 preterm at birth, 30 preterm at expected term age, and 21 term neonates at birth. *Note*: ARA missing in 1 term and 2 preterm. ADP missing in 1 preterm. The bars indicate median and interquartile range. Bonferroni corrected significance level: $P = .004$. Please note that birth versus expected term is paired data, and the remaining is unpaired data. ADP, adenosine diphosphate; ARA, arachidonic acid; CRP, collagen-related peptide; TRAP, thrombin receptor-activating peptide 6

surface markers in preterm platelets at birth compared with term platelets is supported by several previous studies.[29,37,39,40] In accordance with our results, Koltsova et al.[37] found decreased levels of dense granula release (CD63) and α-granula release (P-selectin) at

birth in moderately preterm neonates (GA 32-34 weeks) compared with term neonates, similar to Waller et al,[40] who showed lower levels of bound fibrinogen and P-selectin expression within the first 3 days of life in 20 preterm neonates born with GA <36 weeks

FIGURE 5 Platelet MFI for activation-dependent surface markers bound-fibrinogen, CD63 and P-selectin when activated by agonists collagen-related peptide, adenosine diphosphate, thrombin receptor-activating peptide 6 and arachidonic acid in 43 preterm at birth, 30 preterm at expected term age and 21 term neonates at birth. *Note*: ARA missing in 1 term and 2 preterm. ADP missing in 1 preterm. The bars indicate median and interquartile range. Bonferroni corrected significance level: $p = 0.004$. Please, note that birth vs expected term is paired data and the remaining is unpaired data. Abbreviations: ADP, adenosine diphosphate; ARA, arachidonic acid; CRP, collagen-related peptide; MFI, median fluorescence intensity; TRAP, thrombin receptor-activating peptide 6

compared with terms. Contradicting, Sitaru et al.[29] found no difference in P-selectin levels in 20 preterm neonates with GA 30 to 36 weeks but did find significantly lower levels of P-selectin in 17 preterm neonates born at GA <30 weeks than in term neonates at

birth. Supporting the findings of Sitaru et al, Wasiluk et al.[39] demonstrated a GA-dependent density of P-selectin in preterm neonates with GA 25 to 35 weeks at birth. The postnatal development in platelet activation in preterm neonates is sparsely investigated

TABLE 2 Overview of the results shown in Figures 3-5

	Preterm[a] vs term[b]		Preterm[a] vs expected term age[b]		Expected term age[a] vs term[b]	
	% positive platelets	MFI	% positive platelets	MFI	% positive platelets	MFI
Platelet glycoprotein receptors						
GPIIIa			(↓)	(↑)		(↓)
GPIb						(↓)
GPIa			↓	↓	↑	
GPIIb	↓			↑	↑	↓
GPIX	↓					↓
Platelet activation						
CRP						
Bound fibrinogen	↓		↓	↓		↓
CD63	↓		(↓)	↓		↓
P-selectin	↓		↓	↓		↓
ADP						
Bound fibrinogen	(↓)		(↓)	↑		↓
CD63	(↓)		(↓)	(↑)		↓
P-selectin	↓		↓	↓		↓
TRAP						
Bound fibrinogen	(↓)		(↓)	↓		(↓)
CD63			↓	↓	(↑)	(↓)
P-selectin			↓	↓	↑	(↓)
ARA						
Bound fibrinogen	(↑)		(↑)		↑	↑
CD63					↑	
P-selectin				(↓)	(↓)	

Note: ↓: a is significantly reduced compared with b. ↑: a is significantly increased compared with b. (↓): a is numerically but not significantly reduced compared with b. (↑): a is numerically but not significantly increased compared with b.

Abbreviations: ADP, adenosine diphosphate; ARA, arachidonic acid; CRP, collagen-related peptide; GP, glycoprotein; MFI, median fluorescence intensity; TRAP, thrombin receptor-activating peptide 6.

TABLE 3 Preanalytical activation assessed by expression of P-selectin of term neonatal and preterm neonatal platelets in blood samples

Sample		Preanalytical activation, % positive platelets	Preanalytical activation, MFI	Samples >15% preanalytical activation, n (%)
Blood sample within 24 h				
Preterm neonates	n = 43	12.05 (9.30-22.29)	2.75 (2.24-3.42)	16 (41)
Term neonates	n = 21	14.41 (11.59-19.72)	3.84 (2.92-4.36)	10 (48)
Blood sample at expected term age	n = 30	8.72 (6.11-17.25)	2.50 (2.20-3.11)	8 (27)

Note: Values are presented as median with interquartile range (IQR).
Abbreviation: MFI, median fluorescence intensity.

and results are inconsistent.[29,32,34,37] In this study, most markers of platelet activation in the preterm did not reach term levels within the first 4 to 13 weeks of life, but Michelson et al.[34] and Bednarek et al.[32] found that platelet activation reached adult levels 10 to 14 days after birth. More in line with our results, Koltsova et al.[37] found no increase in α-granula release (P-selectin) during the first 4 days of life. Summarizing, it seems that the reduced platelet activation in preterm neonates is GA dependent and remains reduced at least 3 to 4 days postnatally[29,32,34,37]—most evident in preterm neonates with GA <30 weeks.[29,39] The novelty in this study showing that preterm neonatal platelets do not reach term levels in activation-dependent markers 7.1 weeks after birth is interesting, especially because these neonates display no increased bleeding tendency. One could speculate that this reduced platelet activation in fact is the result of an

age-dependent regulation to avoid excessive platelet activation during processes such as angiogenesis, and therefore potentially this is an evolutionary benefit.[51,52] However, reduced platelet activation in preterm neonates has also raised the question of whether the hyporesponsiveness contributes to the increased risk of bleeding and developing IVH seen in especially the first days of life among preterm neonates born at GA <30 weeks.[5,6,35] Larger clinical studies are needed to investigate if decreased platelet function in preterm neonates is associated with clinical outcomes such as bleeding tendency or risk of IVH or if this is an evolutionary benefit.

In this study, we found some variation in platelet analyses. To some extent, the time span between the first blood sample at birth and blood sampling at expected term could contribute to some of the variation. Further, the preanalytical activation of platelets during collection might contribute to the variation in our results. As we found no difference in the birth samples, we do not expect this to influence the overall conclusion that preterm platelet activation is decreased in preterm neonates compared with term neonates. The blood samples taken at expected term age showed lower preanalytical activation than samples obtained at birth (see Table 3). Even so, the difference in preanalytical activation from the blood samples obtained at birth are not big. Therefore, we do not believe this to affect the overall results, but it might have affected the accuracy of the results.

The strength of the present study is the use of both impedance aggregometry and platelet activation measured by flow cytometry to evaluate platelet function. We evaluated platelet activation after addition of four different agonists (CRP, ADP, TRAP, and ARA), whereas other studies used fewer agonists and investigated fewer activation-dependent markers. Additionally, we included a term neonatal control group instead of comparing preterm neonates with adults. Finally, we investigated platelet function in peripheral blood instead of in umbilical cord blood. However, the present study has limitations. The difficulties in recruiting neonates resulted in a relatively small sample size, and more included neonates born at GA <30 weeks would have been preferable. Further, we did not succeed in obtaining a follow-up sample from all included preterm neonates. The present study was not designed to investigate any potential association between decreased platelet function and bleeding tendency in general or specific diagnoses such as IVH and pulmonary hemorrhages. These outcomes are extremely rare in neonates of GA >28 weeks, and a much larger study would be needed to investigate these associations. The lack of clinical outcomes in the neonates makes it impossible to state the clinical impact of the findings of the present study. Difficulty in collection of blood from the premature neonates might have contributed to the difference in agonist-induced platelet activation reported, which was illustrated by the rather high preanalytical activation. Further, integrin activation was not investigated in this study and therefore cannot be assessed. The purpose of this explorative study was to investigate platelet function in the healthy term and preterm neonate, and the study was not designed to evaluate the pathophysiologic mechanisms that might explain our findings.

5 | CONCLUSION

In conclusion, preterm neonates have a lower platelet count than term neonates at birth, but it increases substantially during the first weeks of life. Preterm neonatal platelet aggregation at expected term age was higher than birth levels and levels in term neonates at birth, probably due to increased platelet count. Preterm neonates demonstrate lower density of the GPIIb and GPIX receptors than term neonates at birth, and the density remains lower 4 to 13 weeks after birth. Finally, preterm platelet activation tends to be decreased at birth compared with term neonates and does not reach term levels during the first 4 to 13 weeks of life.

This study adds knowledge to the understanding and development of platelet function in healthy preterm neonates, but further studies are needed to investigate if the reduced platelet activation contributes to the risk of bleeding and IVH in very preterm neonates.

AUTHOR CONTRIBUTIONS

All authors contributed to the study design, interpreted the results and critically reviewed the manuscript. NTH and AG collected the samples under supervision from AKH. NTH wrote the initial manuscript and did the data analysis. All authors approved the final version of the manuscript to be published.

RELATIONSHIP DISCLOSURE

None declared.

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