Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

# Impaired platelet activity and hypercoagulation in healthy term and moderately preterm newborns during the early neonatal period

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**BACKGROUND:** Preterm newborns are at thrombohemorrhagic risk during the early neonatal period. Taking into account the lack of informative tools for the laboratory diagnosis of hemostasis disorders in newborns, our goal was to determine the baseline values of thrombodynamics and platelet functional activity in healthy term and moderately preterm newborns during the early neonatal period future potential clinical use of these tests.

**METHODS:** Coagulation was assessed using an integral assay of thrombodynamics and standard coagulation assays, and platelet functional activity was estimated by flow cytometry.

**RESULTS:** Hypercoagulation of newborns, represented by a significantly higher clot growth velocity and the presence of spontaneous clots in the thrombodynamics, was combined with platelet hypoactivity. Granule release, phosphatidylserine exposure, and the ability to change shape upon activation were decreased in the platelets of moderately preterm newborns. The platelet function remained at the same level over the first four days of life, whereas the hypercoagulation became less pronounced.

**CONCLUSIONS:** The hemostasis of newborns is characterized by hypercoagulation combined with reduced platelet functional activity. Moderately preterm and term newborns do not differ in the parameters of coagulation, while some of the functional responses of platelets are lower in moderately preterm newborns than in term.

*Pediatric Research* _####################_ ; https://doi.org/10.1038/s41390-018-0184-8

## INTRODUCTION

Preterm newborns are a group at high risk for bleeding and thrombosis in the early neonatal period.[1,2] The most common complications, such as intraventricular hemorrhages, are found in 20% of preterm infants[1] and are associated with increased mortality and adverse neurodevelopment outcomes. Other types of bleeding are less common but may also have fatal consequences.[3] Thrombosis is recorded in 2.4–7% of newborns in intensive care units, more than half of whom are preterm infants. These hemorrhagic and thrombotic manifestations occur mostly during the first week after birth.[4,5]

Despite these well-known alterations in both the plasma and platelet components of hemostasis,[6] thrombotic, and hemorrhagic complications in healthy full-term newborns are rare. It seems that in newborns there exists an alternative balance between procoagulant and anticoagulant components, which appears to be easily affected by complications, including prematurity. The laboratory diagnostic tools at the clinician's disposal are not sufficiently sensitive to the manifestation of impairments in this balance due to the high sensitivity to individual components of the system, and not to the balance as a whole. Measuring individual coagulation protein concentrations cannot serve as an accurate instrument for assessing hemostatic balance due to the complexity and nonlinearity of the system either. Moreover, such measurement is impossible in newborns, as it requires a large amount of blood, which cannot be drawn from these patients. Finally, there exists no routine coagulation test that can reliably reflect the hypercoagulable state. The situation with platelets is also unclear, possibly because the platelet count in peripheral blood is currently the only laboratory criterion on which the attending physician relies in evaluating platelet hemostasis.[7]

Thus, the problem of assessing the balance of neonatal hemostasis still does not have an optimal solution in clinical practice. A suitable solution to this problem seems to be the use of global coagulation assays, which reflect the overall performance of the system,[8] on one hand, and require an acceptable amount of blood for analysis, on the other hand.

Thrombodynamics is a new global hemostasis assay that has been shown to be sensitive to hypercoagulation, to hypocoagulation, and to anticoagulant therapy.[9,10] The thrombodynamics assay is based on the monitoring of spatial fibrin formation initiated by immobilized tissue factor in plasma by video microscopy, so that a clot is initially formed on the activator and then propagates into the plasma. Thrombodynamics is the first laboratory test that takes into account the spatial organization of clotting. In addition, it lacks many of the shortcomings of other

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Received: 10 January 2018 Revised: 29 August 2018 Accepted: 4 September 2018
Published online: 18 September 2018

global tests, such as excessively wide reference ranges in thromboelastography (TEG) or the lack of standardization of thrombin generation test (TGT)[11] while having a sensitivity comparable to that of the TGT and a reproducibility comparable to that of activated partial thromboplastin time (APTT).[12,13]

Taking into account the lack of informative tools for the laboratory diagnosis of hemostasis disorders in newborns, our goal was to determine the baseline values of the platelet functional activity test and thrombodynamics test in groups of healthy term and preterm newborns during the early neonatal period for future potential clinical use of these tests. We also aimed to evaluate whether any significant changes in hemostasis occur during the first days of newborn's life, as these days are the most dangerous in terms of the risk of thrombohemorrhagic complications.

## METHODS

### Study population

This study enrolled moderately preterm newborns with a gestational age of 32–34 complete weeks and a birthweight over 1500 g and term newborns with a gestational age >37 weeks and a birthweight over 2900 g. Gestational age was determined by the last menstruation period. The study was approved by the ethics committee of the center prior to implementation. The ethics committee obliged us to stop recruiting after the obtainment of 10 newborns in the term group who were suitable for analysis. The experimental group consisted of 10 preterm newborns and was formed to obtain statistically equivalent groups. The exclusion criteria were as follows: infants with trauma, congenital anomalies, chromosomal defects, metabolic diseases, congenital heart malformation, infection, thrombosis or bleeding at the moment of inclusion, familial thrombotic or hemorrhagic anamnesis, maternal thrombophilia, Apgar score <7/8 (for term participants), presence of anticoagulant/procoagulant prophylaxis or therapy (for term participants), and infants who received blood products during their first week of life. Patients who developed severe infections or in whom the platelet count decreased to $<40\times10^9$/l were excluded from the study (initially, 13 and 12 patients were recruited into the moderately preterm and term groups, respectively; subsequently, 3 patients and 2 patients, respectively, were excluded from the analysis). All parents gave their written informed consent. Information regarding gestational age, birthweight, maternal anamnesis, and type of delivery and complications until hospital discharge was obtained for all patients. For all preterm newborns, thromboprophylaxis with heparin infusions (30–50 IU/kg/day) was prescribed due to the presence of a central venous catheter.

### Data and specimen collection

Demographic and clinical information was collected from each infant's medical records.

Blood samples were collected at the following two timepoints: on the day of birth (Point 1, P1) and on the third or fourth day of life (Point 2, P2). Blood for blood cell counts was routinely obtained through capillary puncture into one 200-µl K3EDTA tube (Microvette, Sarstedt, Germany) at P1 and P2 for preterm newborns and only at P1 in term newborns. For coagulation assays, at each time point, peripheral venous blood was drawn through the venipuncture into two 1.3-ml nonvacuum tubes (Monovette, Sarstedt, Germany) with 106 mM sodium citrate buffer (pH 5.5) at a 9:1 blood:anticoagulant volume ratio by gravity through a disposable injection sterile Sterican needle 21 G ($0.8\times40$ mm$^2$) (B. Braun, Melsungen, Germany). The blood-draw procedure was standardized for all participants, including adults. A platelet functional activity analysis was performed in 50 µl of whole blood. The remaining blood was processed by centrifugation at $1600\times g$ for 15 min to obtain platelet-poor plasma (PPP). Two hundred microliters of PPP was repeatedly processed by centrifugation at $10{,}000\times g$ for 5 min to obtain 120 µl of platelet-free plasma, which was used for a thrombodynamics assay. The remaining PPP (from 300 to 500 µl depending on the patient's hematocrit) was frozen in liquid nitrogen and stored at −80 °C for the standard coagulation assays (APTT, prothrombin, thrombin time (TT), fibrinogen concentration), antithrombin III (AT III), and protein C concentration determination. An anti-Xa assay was additionally performed to estimate the concentration of heparin in newborns that had central venous lines to receive an unfractionated heparin infusion at very low doses to prevent catheter occlusion.

### Laboratory assays

*Blood cell count and standard coagulation assays*. A blood cell count was performed using Sysmex XS 800i (Sysmex, Kobe, Japan) with Sysmex hematology reagents. Standard coagulation tests including APTT, prothrombin, fibrinogen, AT III, protein C-dimer and D-dimer concentration, and anti-Xa activity were performed using an ACL TOP 700 system and HemosIL® reagents (Instrumentation Laboratory, Bedford, MA, USA).

*Thrombodynamics assay*. A thrombodynamics assay was performed with a thrombodynamics analyzer and a thrombodynamics kit (HemaCore, LLC, Russia) as described.[14] This method is based on registering spatial fibrin clot growth after the activation of clotting in a thin layer of plasma after contact with an immobilized tissue factor-bearing surface. The process of clot growth was registered by serial photos during the test. Based on the photos, a plot of clot growth versus time was obtained (Fig. 1). In some cases, spontaneous clotting (clot formation in the cuvette space not associated with the main clot growth) occurred and was described with a spontaneous clotting plot.[15] The following standard parameters were used: lag time ($T_{lag}$), the delay between the test start and the onset of clot formation; the initial velocity of clot growth ($V_i$), calculated as the mean clot growth velocity over $T_{lag}+2$ min to $T_{lag}+6$; the stationary velocity of clot growth ($V_{st}$), calculated as the mean clot growth velocity over a $T_{lag}+15$ min to $T_{lag}+25$-min interval; the velocity of clot growth ($V$), the parameter calculated as the mean clot growth velocity over the 10-min interval before spontaneous clotting occurs and equal to $V_{st}$ in cases without spontaneous clot formation (for an image of spontaneous clotting see Fig. 1); the density of the clot ($D$), the light scattering intensity of the clot; and the spontaneous clotting time ($T_{sp}$), the time required to fill 5% of the analyzed cuvette area with spontaneous clots.

To obtain the reference ranges for standard coagulation tests, thrombodynamics, and platelet functional activity test, taking into account the blood-draw specificity, we performed an additional analysis on ten healthy adult volunteers, performing the blood draw identically to that of the newborns. Demographic and clinical data on the volunteers are presented in Supplemental Table S1.

*Platelet activity assay*. A platelet activity analysis was performed with the methods described in Suntsova et al.[16] and Ignatova et al.[17] in whole blood instead of platelet-rich plasma. The functional activity was estimated in resting platelets (in order to characterize platelets in the form they exist in the blood flow and to obtain their baseline characteristics) and in activated platelets. Annexin V-Alexa 647 for the evaluation of procoagulant platelets, antibodies against P-selectin (CD62P-Alexa 647) for the evaluation of α-granule expression, CD61-PE and PAC1-FITC antibodies for the evaluation of glycoprotein IIb/IIIa (GPIIb/IIIa) total/activated forms, and CD42b-PE antibody for the evaluation of glycoprotein Ib (GPIb) expression were obtained from Sony Biotechnology (San Jose, CA, USA). Cystein-containing collagen-related peptide used for activating platelets was synthesized by VCPBio Lab (China) and cross-linked as described in ref.[18] All other reagents,

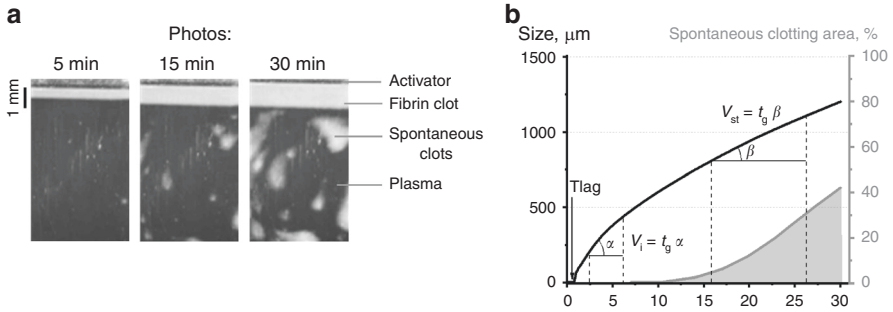


**Fig. 1** Thrombodynamics method principle. **a** Photos of fibrin clot growth. The edge of the activator on the top of the pictures is covered with immobilized tissue factor. The clot starts growing from the edge of the activator to the bulk of the plasma. The process of fibrin clot formation is recorded in a time-lapse video microscopy mode by the dark-field light scattering method. **b** Plot of clot size versus time and plot of spontaneous clotting, representing the thrombodynamics parameters. For a detailed description of test parameters, please see Methods

including mepacrine dihydrochloride for the assessment of dense granule expression, were purchased from Sigma-Aldrich (St Louis, MO, USA).

Statistical analysis and sample size

Statistical analyses were performed using Origin Pro 8 (OriginLab Corp.) software. The median and 5–95 percentile values were used to estimate the assay results. The nonparametric paired-sample Wilcoxon's signed-rank test and Mann–Whitney *U* test were used for the analysis; the significance level was set at $p < 0.05$. A linear correlation analysis was performed using Spearman's rank correlation coefficient. Correlations were considered strong if the correlation coefficient was $R_{Spearman} \geq 0.6$ (or $\leq -0.6$ in case of a negative correlation), and the significance level was set at $p < 0.05$.

## RESULTS

Study population characteristics

A total of ten patients were enrolled into the moderately preterm group (32–34 gestational weeks). Three patients were born by vaginal labor, and seven were born by cesarean section. Some newborns had respiratory distress caused by either transient tachypnea ($n = 3$) or respiratory distress syndrome ($n = 5$). The comparison group consisted of ten healthy term neonates. Eight patients were born by vaginal labor, and two were born by cesarean section. No infants developed clinically significant thrombosis or hemorrhage during the protocol or afterwards. Characteristics of the study populations are summarized in Table 1. Eight neonates in the preterm group received thromboprophylaxis with heparin at a dosage of 50 IU/kg/day and two received thromboprophylaxis with heparin at a dosage of 30 IU/kg/day. Of ten preterm patients, there were two sets of twins and six single preterm births. All participants were of the Caucasian race.

Laboratory assay data

*Standard hematological and coagulation assays.* Blood cell count and standard coagulation values for preterm and term neonatal groups are listed in Table 2. The blood cell counts in both preterm and term neonates varied widely, but most of the values stayed within the normal range for neonates. The platelet count stayed within the normal range for neonates in both investigation groups.

The APTT was prolonged compared to the adult ranges in both neonatal groups during all timepoints of the investigation and did not differ between preterm and term neonates and had a normalization trend from P1 to P2 (Table 2). Prothrombin and fibrinogen values were significantly lower in the neonates and had a normalization trend from P1 to P2. The D-dimer level varied widely and was significantly increased in neonatal samples compared to adult samples. AT III and protein C levels were significantly lower in both neonatal groups (20–30% of the adult amount). The AT III concentration dynamics showed a significant trend towards the adult ranges in both neonatal groups, while the concentration of protein C remained stably low during the time of investigation and was lower in preterm infants in comparison to the term infants at P2. All plasma samples had only traces of heparin (only one neonate had anti-Xa 0.14 IU at P1 of the investigation, while the values of all other neonates remained between 0.00 and 0.03 IU/ml, which lies within the range of instrumental error).

**Table 1.** Demographic and clinical characteristics of the study population

| Median (min–max) | Preterm neonates | Term neonates | *P* value |
|---|---|---|---|
| *N* | 10 | 10 | |
| Gestational age (weeks) | 33 (33–34) | 39 (37–40) | <0.001 |
| Birthweight (g) | 2067 (1492–2660) | 3155 (2940–3820) | <0.001 |
| Height (cm) | 43.5 (41–48) | 51 (48–53) | <0.001 |
| *Apgar score* | | | |
| 1 min | 8 (7–8) | 8 (7–8) | NS |
| 5 min | 8 (8–9) | 9 (8–9) | 0.025 |
| *Delivery type* | | | |
| Vaginal | 3 | 8 | |
| Cesarean section | 7 | 2 | |
| Etiology of prematurity | | | |
| Progressive intrauterine hypoxia | 4 | – | |
| Preterm premature rupture of the membranes (PPROM) | 1 | – | |
| Placental abruption | 1 | – | |
| Spontaneous preterm labor onset | 3 | – | |
| Complications | | | |
| Respiratory distress syndrome | 5 | 0 | |
| Transitory tachypnea | 3 | 0 | |
| No complications | 2 | 10 | |
| Heparin dosage (IU/kg/day) | 50 (0–50) | 0 | |

**Table 2.** Blood cell count and standard coagulation assay results of the patients

| Median (5–95%) | Units | Preterm neonates | | Term neonates | | Reference range for neonates |
|---|---|---|---|---|---|---|
| | | P1 | P2 | P1 | P2 | |
| *Blood cell count* | | | | | | |
| WBC | $10^9$/l | 10.6 (7.8–26.5)[‡] | 11.4 (6.8–14.8) | 23.1 (12.5–31.1) | N/A | 5.9–17.5 |
| RBC | $10^{12}$/l | 5.1 (4.1–6.1) | 4.5 (3.6–5.4) | 5.4 (4.5–6.9) | N/A | 3.9–5.9 |
| HGB | g/l | 183.5 (160–205) | 169.5 (125–192) | 193 (169–237) | N/A | 134–198 |
| HCT | % | 50.2 (43.6–60.8) | 44.0 (36.2–55.1) | 51.3 (46.6–60.7) | N/A | 41.0–65.0 |
| PLT | $10^9$/l | 268.5 (158–375) | 230 (185–359) | 307 (158–429) | N/A | 144–449 |
| *Coagulation assays* | | | | | | Adult values |
| APTT | s | 64.8 (37.1–167.8)* | 49.8 (35.7–60.5)*,[§] | 50.0 (38.5–67.7)* | 38.5 (33.1–55.9)*,[§] | 32.3 (28.2–36.6) |
| Prothrombin | % | 71 (49–96)* | 87 (68–104)[§] | 69.5 (58–77)* | 79.5 (72–100)*,[§] | 98 (87–108) |
| Fibrinogen | g/l | 1.47 (0.96–2.66)* | 2.57 (1.48–4.49)[§] | 1.63 (1.28–2.55)* | 2.81 (1.63–3.28)[§] | 2.27 (1.75–3.28) |
| D-dimer | ng/ml | 399.5 (48–36,915)*,[‡] | 370.5 (154–12,956)* | 1556 (504–39,102)* | 603 (258–4674) | 51 (15–98) |
| AT III | % | 34 (13–52)* | 49 (21–73)*,[§] | 39 (28–57)* | 56.5 (34–66)*,[§] | 109 (89–139) |
| Protein C | % | 18 (8–37)* | 19.5 (11–36)*,[‡] | 26.5 (18–54)* | 33.5 (17–57)* | 88 (57–128) |
| Anti-Xa | IU/ml | 0.00 (0.00–0.14)*,[‡] | 0.00 (0.00–0.03) | 0.00 (0.00–0.00) | 0.00 (0.00–0.03) | 0 (0.00–0.00) |

Point 1, P1 first day after birth, Point 2, P2 third or fourth day after birth, N/A results of the blood cell count for term newborns at P2 are not available because, in this group, it is routinely performed only once on the day of birth
*Significantly differs from adults, $p < 0.05$, Mann–Whitney test; [‡]significantly differs from term infants (for preterm group), $p < 0.05$, Mann–Whitney test; [§]significantly differs from P1, $p < 0.05$, Wilcoxon's pair signed-rank test

*Thrombodynamics assay*. $T_{lag}$ in the thrombodynamics assay was significantly shorter in both newborn groups than in adults ($p < 0.05$) (Fig. 2a). Parameters $V_i$ and $V$, which indicated the clot growth rate, were increased significantly compared to the adult values ($p < 0.05$) (Fig. 2b, c). The term newborn group had a significant tendency towards normalization at P2. Some of the patients showed marked hypercoagulation with spontaneous clotting (Fig. 2e, f). At P1, most of the samples were characterized by the presence of spontaneous clots (44% of the preterm group and 80% of the term group), which disappeared at P2 (only 20% of the term group had spontaneous clotting). Parameter $D$, indicating the clot density, was increased significantly at P2 compared to P1 in both newborn groups ($p < 0.05$) (Fig. 2d), which remained in correlation with the amount of fibrinogen.[19] Numerical data regarding the thrombodynamics are provided in Supplemental Table S2.

The only interassay correlation observed was between parameter $D$ and the fibrinogen amount ($R_{Spearman} = 0.93$, $p < 0.001$) (Fig. S1A).

*Platelet function assay*. The expression of GPIb was significantly higher in the platelets of both newborn groups in both the resting (Fig. 2a) and activated states ($p < 0.05$) (Fig. 3b) than in adult platelets. The GPIb expression did not differ between term and preterm newborns. The expression of total GPIIb/IIIa was significantly increased in the term infants' resting platelets ($p < 0.05$) (Fig. 3c), while the values of the preterm newborns did not differ significantly from those of the adults and were significantly decreased compared to those in term newborns ($p < 0.05$). However, after activation, the expression of total GPIIb/IIIa did not differ between term newborns and adults (Fig. 3d), while the values in preterm newborns were lower than those in term newborns and adults. The expression of GPIIb/IIIa in the activated form in resting platelets did not differ between groups (Fig. 2e) and did not reveal any preactivation. Active GPIIb/IIIa on activated platelets was significantly lower in both newborn groups than in adults ($p < 0.05$) and did not differ between term and preterm newborns (Fig. 3f). None of the integrin expression levels changed from P1 to P2. Some of the preterm newborns had increased amounts of circulating procoagulant platelets prior to activation at P1 (Fig. 3g). The number of procoagulant platelets after activation was significantly lower in samples of preterm newborns than in adult samples ($p < 0.05$) (Fig. 3h). The number of procoagulant platelets in term infants was lower than that in adults, but the difference was nonsignificant. Platelet size was decreased in both preterm and term newborns (Table S3). The change in the shape of platelets after activation was evaluated by calculating the ratio of forward light scattering (FSC) values in resting/activated platelets. The change in FSC was significantly smaller in both term and preterm newborn samples than in adult samples ($p < 0.05$) and was significantly lower in preterm samples than in term samples ($p < 0.05$) (Fig. 3i). The FSC change in the term newborns' platelets demonstrated a significant trend towards adult values from P1 to P2 and did not differ significantly from the adult values at P2, while the preterm newborns' platelet FSC ratio remained lowered during the investigation period (Fig. 3i). We performed an additional analysis of the GPIb expression ratio, which was calculated similarly to the FSC ratio. The analysis demonstrated similar results, although due to the wider variability of the adult values, the difference between groups remained nonsignificant (Fig. 3j).

The number of dense granules in either term or preterm neonatal platelets before activation estimated by mepacrine uptake was decreased by approximately 40% in comparison to that in adult platelets (Fig. 4a). The release of dense granules after platelet activation estimated as a shift in mepacrine accumulation (the difference between mepacrine uptake by activated platelets and mepacrine uptake by resting platelets) was also decreased in both neonatal groups and was lower in preterm newborns in comparison to that in term newborns at P1 (Fig. 4b). The amount of P-selectin on the surface of circulating neonatal and adult platelets was once again comparable and low (Fig. 4c), but the amount after activation was almost 50% lower in the neonatal samples after the activation. The amount of P-selectin was also decreased in the preterm group in comparison with that in the term neonates, indicating the depressed secretory functions of

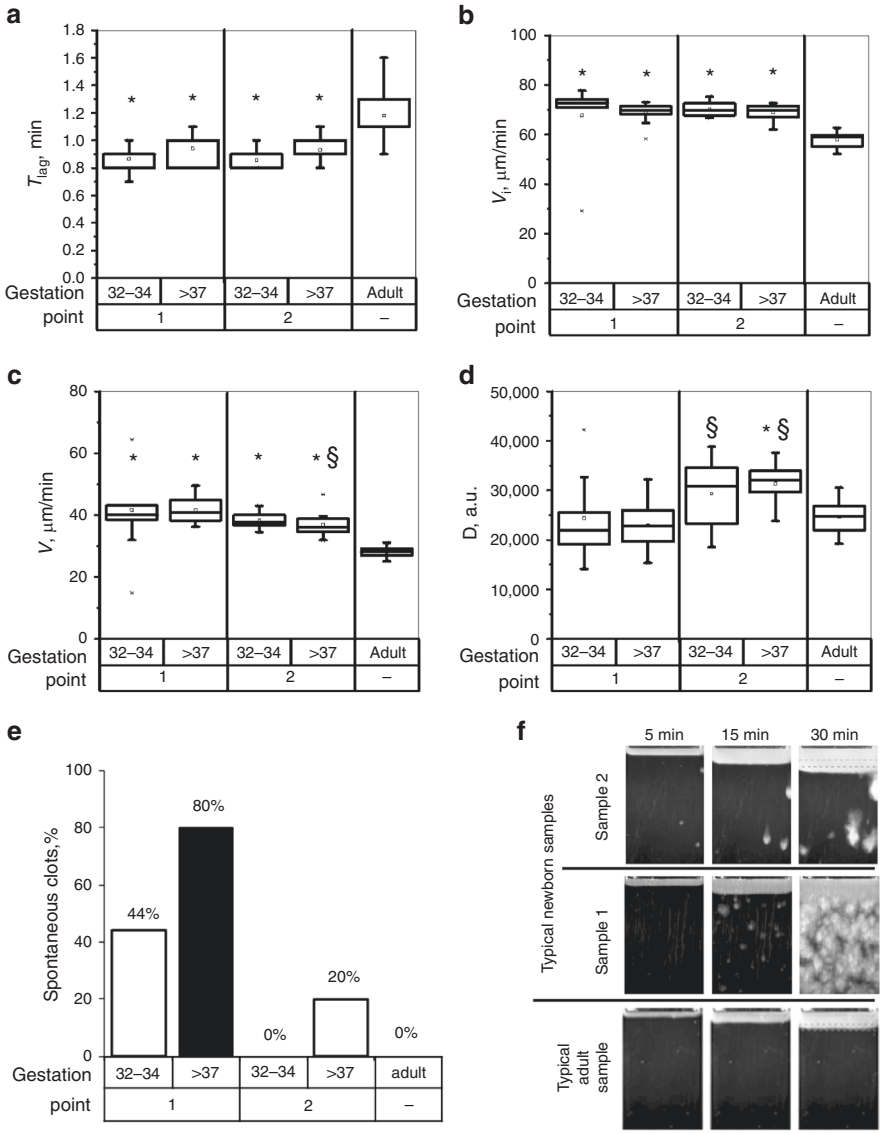


**Fig. 2** Thrombodynamics test parameters. Patients were divided into groups according to gestational age (10 preterm neonates, 10 term neonates). Samples of 10 adult healthy volunteers were used as a control. **a** $T_{lag}$ (min); **b** $V_i$ (μm/min); **c** $V$ (μm/min); **d** $D$ (a.u.).; **e** percentage of samples with the presence of spontaneous clots; **f** photos of fibrin clot formation in newborn and adult plasmas. Here and further, the box plots indicate the following parameters: the mean value (the dot inside the box), the median (the horizontal line inside the box), the 25th and 75th percentiles (the bottom and top of the box, respectively), and the outliers (the ends of the whiskers). *Significantly differs from adults, $p < 0.05$, Mann–Whitney test; ‡significantly differs from term infants (for preterm group), $p < 0.05$, Mann–Whitney test; §significantly differs from P1, $p < 0.05$, Wilcoxon's pair signed-rank test

newborn platelets (Fig. 4d). In the term neonates, but not in the preterm neonates, the amount of P-selectin began to improve by P2. Numerical data regarding the platelet functional activity can be obtained from Supplemental Table S3.

The platelet size estimated by FSC was negatively correlated with the platelet count in both resting and activated states (for resting platelets, $R_{Spearman} = -0.68$, $p < 0.001$; for activated platelets, $R_{Spearman} = -0.82$, $p < 0.001$) (Fig. S1B, C). No other platelet activity test parameters were correlated with the platelet count or any coagulation parameters.

## DISCUSSION

The estimated standard coagulation tests results and anticoagulant concentrations are consistent with the previous studies.[20,21] In our study, neonatal coagulation was characterized by markedly prolonged coagulation times, decreased fibrinogen concentrations, decreased concentrations of natural anticoagulants and high D-dimer levels. High D-dimer in newborns levels may reflect activation of fetal fibrinolysis following labor[22] or might represent low-grade activation of the coagulation cascade, as a result of the circulatory adjustments involving closure of the ductus venosus and ductus arteriosus after birth.[23]

Our study is the first to implement a thrombodynamics test to evaluate coagulation in neonates. The thrombodynamics parameters showed hypercoagulation, which persisted during the first days of life of both term and preterm infants, with a tendency to normalization in term newborns. Other global hemostasis tests, such as TGT and TEG show multidirectional changes in newborn blood: TGT reveals a decreased thrombin potential or values within the adult normal range in the presence of soluble thrombomodulin,[24] while TEG reveals hypercoagulation[25] or normal coagulation.[26] This relative procoagulant state in the newborn has been attributed to an altered balance of

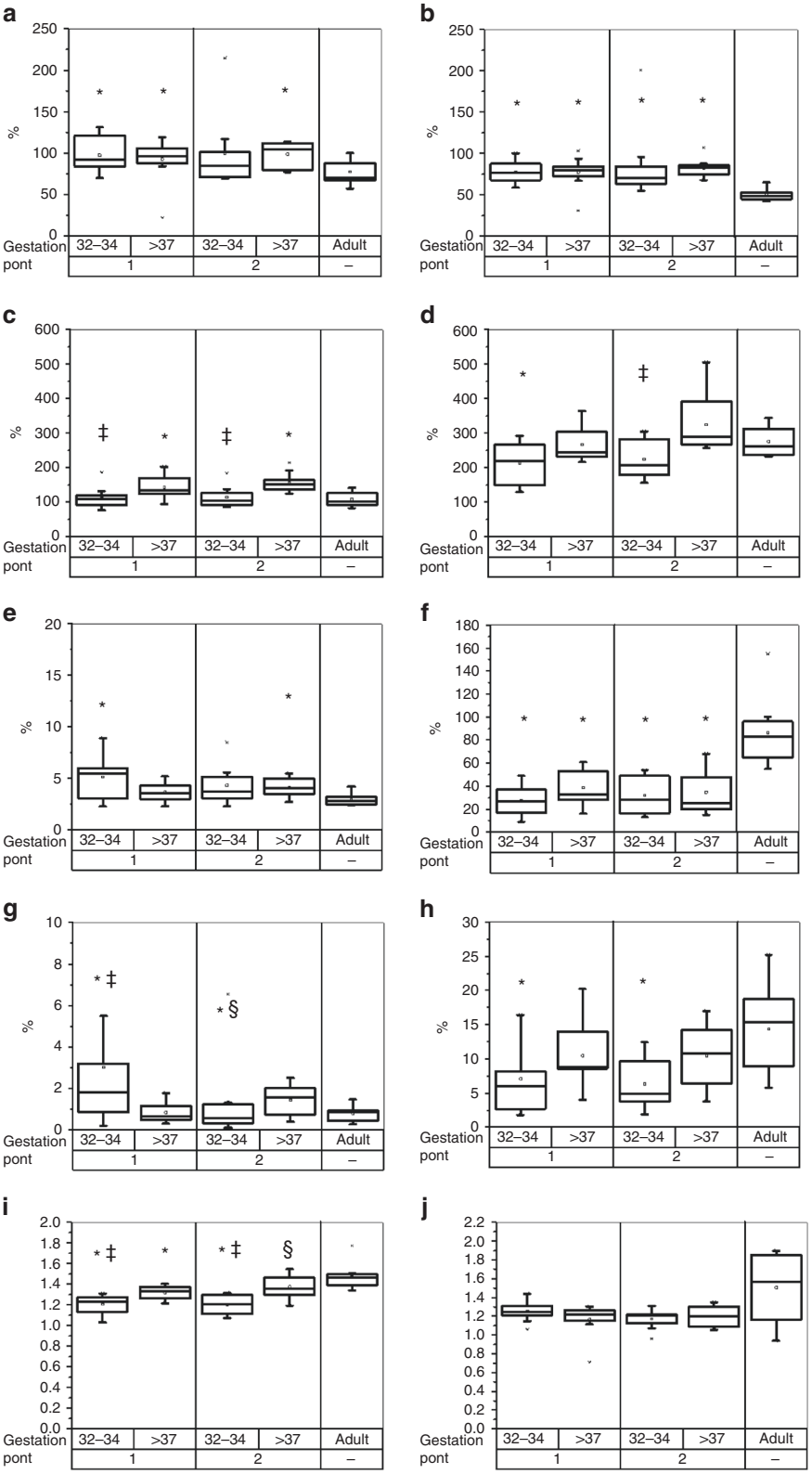


**Fig. 3** Parameters of the platelet functional activity (glycoprotein expression, procoagulant platelets, shape change parameters). Patients were divided into groups according to gestational age. Platelets of 10 preterm neonates, 10 term neonates, and 10 adult healthy donors were characterized by flow cytometry before and after stimulation. **a**–**g** represents resting platelets, **b**–**h** represents activated platelets. **a**, **b** Level of GP I expression; **c**, **d** level of total GPIIb/IIIa expression; **e**, **f** level of activated GPIIb/IIIa expression; **g**, **h** percentage of procoagulant platelets; **i** forward light scattering (FSC) change (FSC resting/FSC activated); **j** GP I expression ratio (GP I resting/GP I activated). *Significantly differs from adults, $p < 0.05$, Mann–Whitney test; ‡significantly differs from term infants (for the preterm group), $p < 0.05$, Mann–Whitney test; §significantly differs from P1, $p < 0.05$, Wilcoxon's pair signed-rank test

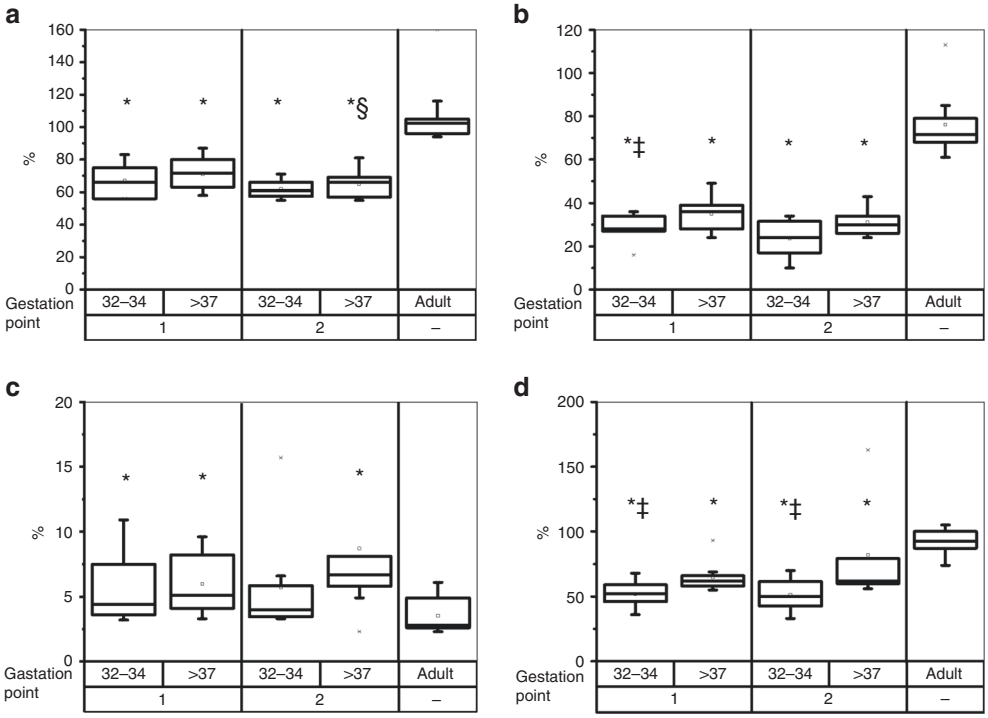


**Fig. 4** Parameters of the platelet functional activity (granule release). Patients were divided into groups according to gestational age. Platelets of 10 preterm neonates, 10 term neonates, and 10 adult healthy donors were characterized by flow cytometry before and after stimulation. **a** Dense granule amount before platelet activation; **b** Dense granules release after platelet activation; **c** P-selectin of α-granules before activation; **d** P-selectin of α-granules after activation. *Significantly differs from adults, $p < 0.05$, Mann–Whitney test; ‡significantly differs from term infants (for preterm group), $p < 0.05$, Mann–Whitney test; §significantly differs from P1, $p < 0.05$, Wilcoxon's pair signed-rank test

anticoagulant and procoagulant factors.[25] Spontaneous clotting in thrombodynamics can be caused by preactivation of coagulation via circulating TF in the newborn blood circulation, which might be related to the traumatic effect of delivery and cutting of the umbilical cord, causing the release of this protein from the injured tissues and the endothelium.[27] This finding may be explained by the fact that thrombodynamics is particularly sensitive to both hypercoagulation and anticoagulant treatment,[9,14,15,28] which makes it an efficient tool for coagulation assessments in patient groups with systemic hypercoagulation. The assay allows obtaining information about the coagulation system, which is fundamentally inaccessible with the existing routine hemostasis coagulation assays and provides a quantitative estimation of all physiological stages of the fibrin clot growth.

Taking into account the presence of low-dose heparin infusion in preterm newborns, we performed an anti-Xa activity test to estimate the amount of heparin and justify the comparison of the term and preterm groups. In both preterm newborns (receiving heparin) and term newborns (not receiving heparin), the anti-Xa activity varied within 0.00–0.03 IU/ml. None of the tests used is sensitive to such small concentrations of heparin, so we considered the comparison of the groups appropriate.

Our data on the impaired platelet functional activity is consistent with the results from most of the previous studies on newborns.[29–31] However, there are no studies in which the functional responses of platelets from term infants and premature newborns have been systematically compared. We show that moderately preterm infants do not differ from term infants in GPIb, P-selectin release, or mepacrine uptake/release, but have decreased GPIIb-IIIa, lower GPIIb-IIIa activation, and PS externalization upon stimulation (but a remarkably higher circulating PS exposure possibly linked to their hypercoagulation). Interestingly, preterm infants do not differ from the term infants in their dynamics: all parameters in P2 are similar to those in P1, except for decreased circulating PS in preterm newborns, which is normalized by P2. The most accepted view is that platelet hypoactivity in newborns arises from the general immaturity of thrombopoiesis and can be specifically linked to deficiencies of phospholipid metabolism and thromboxane production, different regulation of GPIIb/IIIa activation, impaired mobilization of calcium and intracellular signaling, granule secretion, and aggregation.[32]

We observed an interesting negative correlation between the platelet size of both resting and activated platelets and the platelet count. This finding is consistent with that of Canpolat et al.[33] However, there is no clear explanation for this phenomenon, except for the general immaturity of hematopoiesis in neonates.

Neonatal platelets were significantly smaller than adult ones, which is well known from the literature.[34] However, we observed that not only the platelet size but also the neonatal platelet shape changes after activation (as indicated by the FSC change upon activation) was less pronounced than those in adult platelets. This shift resolved in term infants by the third day of life but was more pronounced and persisted in preterm infants during the entire investigation time. This fact may indirectly indicate the difference between the ultrastructure of activated platelets of adults and newborns. Our data are consistent with those of Saving et al.[35] However, the blood-draw method and sample preparation that were used in this study dramatically differs from ours, and thus the apparent correlation of the results should be perceived with a certain degree of caution.

In conclusion, our major novel findings include the following facts:

(1) platelet hypoactivity in both preterm and term newborns (interestingly, without any trace of preactivation) is unexpectedly combined with significant hypercoagulation detected with thrombodynamics, observed to the same

degree in both groups;
(2) all platelet functional responses (specifically, granule release, PS exposure, and ability to change the shape upon activation) are decreased in preterm newborns compared to terms;
(3) in both preterm and healthy newborns, platelet function defects remain at the same level over the first four days of life, while hypercoagulation in thrombodynamics becomes less pronounced.

Taking into account the decrease in the platelet functional activity of newborn platelets and hypercoagulation (which can be derived from either an impaired balance between coagulation factor precursors and natural anticoagulants or the presence of activated factors in the blood flow), we may speculate that the balance between platelet and plasma hemostasis in newborns may be altered both in newborns in general compared to adults and in preterm newborns compared to term newborns and adults.

Developmental hemostasis is a concept that is now well accepted among experts in the field of hemostasis, but which is potentially underrecognized by many clinicians and scientists working in the field of medicine and diagnostics.[36] Our results from full-term newborns are in good agreement with this concept. At the same time, we introduced two significant additions. First, we evaluated the integral state of plasma hemostasis with thrombodynamics. Until now, only the TGT has been used for these purposes. However, it is well known that the generation of thrombin in the TGT depends linearly on the concentration of prothrombin.[37] This feature is because the test measures not the final response of the system (a clot), but the regulatory protein (thrombin). It is well known that fibrin appears in the presence of 5–10 nM thrombin.[38] Thus, the hypocoagulation in the TGT in newborns is a consequence of specific changes in their plasma components and cannot be informative from the viewpoint of the global response of the hemostatic system. Thrombodynamics allows the researcher to evaluate the characteristics of fibrin clot growth and clearly shows increased coagulation in newborns. We believe that this condition is due to a violation of the balance of procoagulation and anticoagulation proteins. Further study is required to prove these speculations and to evaluate neonatal hemostasis responses in disease states. The physiological causes responsible for the observed assay results in neonates remain to be elucidated and are of tremendous biological significance.[39]

It is worth noting that the results of the current study can be implemented only to moderately preterm infants and the results may differ for groups of more premature infants. The study of hemostasis of very and extremely premature newborns is a task of great importance, since the hemorrhagic and thrombotic complications are much more common in this population. However, the recruitment of this group for global hemostasis assays testing is complicated, since blood sampling for analysis from the peripheral vein in these newborns is virtually impossible. We are currently working at the subject and hope to provide the data on thrombodynamics and platelet function of very and extremely preterm newborns in near future. We decided to conduct a general investigation, and observing the clinical outcomes was beyond the scope of the current study. Further work in this direction must consist of the identification of clinical risk groups that can benefit from the screening of hemostasis during the early days of life and the development of strategies for anticoagulant and procoagulant prophylaxis and therapy in such patients.

## CONCLUSIONS

The hemostasis of newborns is characterized by pronounced hypercoagulation and reduced functional activity of their platelets in comparison with the platelets obtained from adults. Preterm and term newborns do not differ in the parameters of coagulation; however, some of the functional responses of platelets are lower in preterm infants than in term infants. The obtained data can serve as a basis for developing a complex of tests for monitoring the state of hemostasis in newborns.

## ACKNOWLEDGEMENTS

The online version of this article (https://doi.org/10.1038/s41390-018-0184-8) contains supplementary material, which is available to authorized users.

## FUNDING

This work was supported by a grant from the Russian Science Foundation (17-74-10224) to A.N.B.

## AUTHOR CONTRIBUTION

E.M.K., E.N.B., M.A.P., and A.N.B. designed the study. E.N.B., O.V.I., A.R.K., A.A.L., L.A.T., V. V.Z., and D.N.D. recruited the participants and collected the clinical data from the participants. E.M.K. and A.D.K. performed the thrombodynamics assay, A.A.I. and D.M. P performed the platelet functional testing, and A.V.P. performed the other coagulation assays. E.M.K. performed the statistical analysis. E.M.K. wrote the manuscript, which was critically revised by A.N.B., E.N.B., and M.A.P. and finally approved by G.T.S. and F.I.A.

## ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-018-0184-8) contains supplementary material, which is available to authorized users.

**Competing interests:** F.I.A. is former employee and founder of HemaCore LLC, which holds several patents and patent applications that are related to the diagnostic use of spatial clot growth and has developed an assay under the trade name of Thrombodynamics®.



## REFERENCES

1. Szpecht, D., Szymankiewicz, M., Nowak, I. & Gadzinowski, J. Intraventricular hemorrhage in neonates born before 32 weeks of gestation-retrospective analysis of risk factors. *Childs Nerv. Syst.* **32**, 1399–1404 (2016).
2. Bhat, R. & Monagle, P. The preterm infant with thrombosis. *Arch. Dis. Child Fetal Neonatal Ed.* **97**, F423–F428 (2012).
3. Yum, S. K. et al. Risk factor profile of massive pulmonary haemorrhage in neonates: the impact on survival studied in a tertiary care centre. *J. Matern. Fetal Neonatal Med.* **29**, 338–343 (2016).
4. van Elteren, H. A. et al. Management and outcome in 32 neonates with thrombotic events. *Int. J. Pediatr.* 2011;217564. https://doi.org/10.1155/2011/217564. Epub 2011 Aug 11
5. Tavil, B. et al. Foetal and neonatal intracranial haemorrhage in term newborn infants: Hacettepe University experience. *Blood Coagul. Fibrinolysis* **27**, 163–168 (2016).
6. Ziegler, B., Solomon, C., Cadamuro, J. & Jones, N. Thromboelastometric monitoring of the hemostatic effect of platelet concentrates transfusion in thrombocytopenic children undergoing chemotherapy. *Clin. Appl. Thromb. Hemost.* **21**, 558–564 (2015).
7. Deschmann, E., Sola-Visner, M. & Saxonhouse, M. A. Primary hemostasis in neonates with thrombocytopenia. *J. Pediatr.* **164**, 167–172 (2014).
8. Panteleev, M. A. & Hemker, H. C. Global/integral assays in hemostasis diagnostics: promises, successes, problems and prospects. *Thromb. J.* **13**, 5 (2015).
9. Gracheva, M. A. et al. Thromboelastography, thrombin generation test and thrombodynamics reveal hypercoagulability in patients with multiple myeloma. *Leuk. Lymphoma* **56**, 3418–3425 (2015).
10. Seregina, E. A. et al. Laboratory tests for coagulation system monitoring in a patient with β-thalassemia. *Int J. Hematol.* **99**, 588–596 (2014).
11. Sinauridze, E. I. et al. Thrombodynamics, a new global coagulation test: measurement of heparin efficiency. *Talanta* **180**, 282–291 (2018).

12. Sinauridze, E. I. et al. Hypercoagulation, caused by the dilution of plasma by volume expanders. *Technol. Living Syst.* **5**, 3–14 (2008).
13. Gracheva, M. A., Balandina, A. N., Ataullakhanov, F. I. & Roitman, E. V. Comparison of responses of integrated and screening tests for hemostasis assessment to various coagulation statesin models in vitro. *Thromb. Haemost. Rheol.* **4**, 64–71 (2016).
14. Koltsova, E. M. et al. The laboratory control of anticoagulant thromboprophylaxis during the early postpartum period after cesarean delivery. *J. Perinat. Med* **46**, 251–260 (2018).
15. Lipets, E. et al. Circulating contact-pathway-activating microparticles together with factors IXa and XIa induce spontaneous clotting in plasma of hematology and cardiologic patients. *PLoS ONE* **9**, e87692 (2014).
16. Suntsova, E. V. et al. Bleeding tendency and platelet function during treatment with romiplostim in children with severe immune thrombocytopenic purpura. *Int J. Hematol.* **105**, 1–8 (2017).
17. Ignatova, A. A., Karpova, O. V., Trakhtman, P. E., Rumiantsev, S. A. & Panteleev, M. A. Functional characteristics and clinical effectiveness of platelet concentrates treated with riboflavin and ultraviolet light in plasma and in platelet additive solution. *Vox Sang.* **110**, 244–252 (2016).
18. Morton, L. F., Hargreaves, P. G., Farndale, R. W., Young, R. D. & Barnes, M. J. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem. J.* **306**, 337–344 (1995).
19. Ovanesov, M. V., Ananyeva, N. M., Panteleev, M. A., Ataullakhanov, F. I. & Saenko, E. L. Initiation and propagation of coagulation from tissue factor-bearing cell monolayers to plasma: Initiator cells do not regulate spatial growth rate. *J. Thromb. Haemost.* **3**, 321–331 (2005).
20. Andrew, M. et al. Development of the human coagulation system in the full-term infant. *Blood* **70**, 165–172 (1987).
21. Andrew, M. et al. Development of the human coagulation system in the healthy premature infant. *Blood* **72**, 1651–1657 (1988).
22. Murtha, A. P., Boggess, K. A., Jimmerson, C. E., Greig, P. C. & Herbert, W. N. Umbilical venous D-dimer concentrations with and without labor. *Obstet. Gynecol.* **92**, 184–186 (1998).
23. Hudson, I. R. et al. Increased concentrations of D-dimers in newborn infants. *Arch. Dis. Child* **65**, 383–384 (1990).
24. Tripodi, A. et al. Normal thrombin generation in neonates in spite of prolonged conventional coagulation tests. *Haematologica* **93**, 1256–1259 (2008).
25. Sewell, E. K. et al. Thromboelastography in term neonates: an alternative approach to evaluating coagulopathy. *Arch. Dis. Child Fetal Neonatal Ed.* **102**, F79–F84 (2017).
26. Kettner, S. C. et al. Heparinase-modified thrombelastography in term and preterm neonates. *Anesth. Analg.* **98**, 1650–1652 (2004).
27. Tay, S. P., Cheong, S. K. & Boo, N. Y. Circulating tissue factor, tissue factor pathway inhibitor and D-dimer in umbilical cord blood of normal term neonates and adult plasma. *Blood Coagul. Fibrinolysis* **14**, 125–129 (2003).
28. Soshitova, N. P. et al. Predicting prothrombotic tendencies in sepsis using spatial clot growth dynamics. *Blood Coagul. Fibrinolysis* **23**, 498–507 (2012).
29. Sitaru, A. G. et al. Neonatal platelets from cord blood and peripheral blood. *Platelets* **16**, 203–210 (2005).
30. Urban, D. et al. Decreased numbers of dense granules in fetal and neonatal platelets. *Haematologica* **102**, e36–8 (2017).
31. Mankin, P., Maragos, J., Akhand, M. & Saving, K. L. Imparied platelet-dense granule release in neonates. *J. Pediatr. Hematol. Oncol.* **22**, 143–147 (2000).
32. Del Vecchio, A., Motta, M. & Romagnoli, C. Neonatal platelet function. *Clin. Perinatol.* **42**, 625–638 (2015).
33. Canpolat, F. E., Yurdakök, M., Armangil, D. & Yiit, Ş. Mean platelet volume in neonatal respiratory distress syndrome. *Pediatr. Int* **51**, 314–316 (2009).
34. Wiedmeier, S. E., Henry, E., Sola-Visner, M. C. & Christensen, R. D. Platelet reference ranges for neonates, defined using data from over 47 000 patients in a multi-hospital healthcare system. *J. Perinatol.* **29**, 130–136 (2009).
35. Saving, K. L., Jennings, D. E., Aldag, J. C. & Caughey, R. C. Platelet ultrastructure of high-risk premature infants. *Thromb. Res* **73**, 371–384 (1994).
36. Monagle, P. et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb. Haemost.* **95**, 362–372 (2006).
37. Al Dieri, R. et al. The thrombogram in rare inherited coagulation disorders: Its relation to clinical bleeding. *Thromb. Haemost.* **88**, 576–582 (2002).
38. Mann, K., Brummel, K. & Butenal, S. What is all that thrombin for? *Thromb. Haemost.* **1**, 1504–1514 (2003).
39. Bauman, M. E., Cheung, P. Y. & Massicotte, M. P. Hemostasis and platelet dysfunction in asphyxiated neonates. *J. Pediatr.* **158**, e35–e39 (2011).