Prostaglandin E\(_2\) levels and platelet function are different in cord blood compared to adults

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**Summary**

Neonatal platelets support primary haemostasis and thrombin generation as well as adult platelets, despite observable hypoaggregability in vitro. High prostaglandin E\(_2\) levels at accouchement could account for inhibited platelet function via the EP\(_2\) receptor. We set out to determine prostaglandin E\(_2\) plasma levels in cord blood of healthy neonates and evaluate the impact of prostaglandin E\(_2\) on platelet function in adult and cord blood samples. Prostaglandin E\(_2\) plasma levels were measured in cord blood and venous adult blood using GC-MS. Impact of prostaglandin E\(_2\) on platelet aggregation was measured by spiking cord blood and adult samples. Contributions of EP\(_3\) and EP\(_4\) receptors were evaluated using respective antagonists. Intracellular cAMP concentrations were measured using a commercial ELISA-kit. Prostaglandin E\(_2\) plasma levels were substantially higher in cord blood than in adult samples. Spiking with prostaglandin E\(_2\) resulted in a slight but consistent reduction of platelet aggregation in adult blood, but response to PGE\(_2\) was blunted in cord blood samples. Aggregation response of spiked adult samples was still higher than with non-spiked cord blood samples. Blockage of EP\(_2\) receptors resulted in improved platelet aggregation in adult platelets upon prostaglandin E\(_2\) spiking, while aggregation in cord blood samples remained unaltered. Intracellular cAMP concentrations after preincubation with prostaglandin E\(_2\) were only increased in adult samples. In conclusion, very high prostaglandin E\(_2\) concentrations in cord blood affect platelet function. This effect may partially explain neonatal platelet hypoaggregability. Peak levels of prostaglandin E\(_2\) can potentially protect against birth stress-induced platelet activation.

**Keywords**

Cyclic AMP, neonate, prostaglandin E\(_2\), prostanoid receptor EP4, platelet aggregation

**Introduction**

Neonatal platelets show distinct functional deficiencies when compared to adult platelets that are even stronger in preterm neonates (1–4). Platelet counts and size in healthy neonates are comparable to adult controls (5, 6). Certain differences have been observed in the functional structure of neonatal platelets: Surface expression of GPIbα (CD42b), a receptor for von Willebrand factor was found to be lower in neonatal platelets, and there seems to be a correlation between P-selectin expression and gestational age (7–10). The results for integrin αIIbβ3, a receptor for fibrinogen, fibronectin and von Willebrand factor, vary depending on the study (8, 9).

Some functional studies of platelet aggregation in neonates have been performed, and all results point to hypoaggregability of neonatal platelets in vitro with many commonly used agonists such as ADP, epinephrine, thrombin, and thromboxane analogs (2–4).

It has been argued that degranulation of neonatal platelets occurs during delivery and that platelets become refractory and consequently hyporesponsive to potent agonists. However, in agreement with other research groups we could find no signs of partial activation of neonatal platelets during delivery (11–13).

Reasons for this hyporesponsiveness of neonatal platelets vary depending on the agonist. The number of thromboxane receptors is similar on neonatal and adult platelets; however, thromboxane A2-response and resulting synthesis is impaired in neonates due to reduced GTPase activity of the Gaq-subunit coupled to the receptor (14). Response to collagen has been shown to be reduced due to impaired mobilisation of intracellular calcium (15), and there is approximately half the number of α2-adrenergic receptors present on neonatal platelets compared to adult platelets, accounting for the hyporesponsiveness to epinephrine (16).

Ristocetin-induced aggregation is higher in neonatal platelet-rich plasma than in adult controls, caused by larger multimeric vWFP in nonates (17). Our group could show that there really is a higher ristocetin and shear stress-induced binding of this larger multimeric vWFP to neonatal as well as to adult platelets (18).

Despite this known hyporeactivity, neonatal primary haemostasis in vivo does not seem to be impaired. Neonates are not prone...
to easy bruising, and bleeding times as well as PFA-100 closure times are shorter than adult control values (19–21).

Investigating the role of platelets in secondary haemostasis, we also observed a discrepancy: We showed that newborn platelets support thrombin generation as well as adult platelets (22). This is extraordinary because responsiveness to thrombin is necessary for initiating the haemostasis process through various mechanisms. We identified lower levels of protease-activated receptors 1 and 4 in neonatal platelets of cord blood as potential cause for their hypoaggregatability to thrombin, but did not find a compensatory mechanism accounting for the well functioning support of thrombin generation (23).

Recent investigations on the closure of the ductus arteriosus led to the identification of platelets as an important contributing factor (24). The ductus arteriosus is a shunt pathway between the fetal pulmonary artery and the aortic arch that closes within the first weeks after birth in a process dependent on several mediators acting in synergy (25). In the fetal stage the ductus arteriosus is actively kept open by high blood concentrations of prostaglandin E2 (PGE2), a potent vessel relaxant. The placenta has been identified to be a major source for high circulating PGE2 levels (26, 27). Toward the end of gestation there is a significant increase of PGE2 concentration detectable, followed by a steep decline after birth (28).

The succeeding ischaemic stimulus initiates a process that fundamentally resembles the formation of atherosclerotic lesions or neointima formation after vascular injury (29, 30). It could be shown that in mice platelets adhere to the residual lumen of the constricted ductus arteriosus within minutes after birth, and occlude it to form a complete functional closure (24). The authors of this study postulate that very high concentrations of PGE2 in the fetal circulation could exert an inhibitory stimulus on fetal and neonatal platelet function that rapidly decreases within minutes after accouchement (31). This hypothesis could explain some of the discrepancies mentioned above.

In vitro experiments with adult platelets show that low concentrations of PGE2 enhance overall platelet sensitivity by decreasing the intracellular cyclic adenosine monophosphate (cAMP) concentration via EP2 signalling. On the other hand, very high concentrations can impair platelet signalling by increasing intracellular cAMP concentration (32). The responsible receptor for PGE2-induced platelet inhibition was postulated to be the prostanoid receptor (IP1), but has been identified later as the EP2 receptor (33–35).

However, the concentrations of PGE2 used to demonstrate an inhibiting effect are much higher than expected physiological values in fetuses and neonates. Normal ranges for plasma concentrations of PGE2 at birth need to be established to test if these ranges are enhancing or inhibiting neonatal platelet function. Furthermore, it is not granted that the reaction of neonatal platelets to the eicosanoid is comparable to platelets from adults. Receptor levels could be lower or signalling pathways could be impaired.

Our goal was to establish a reference range for PGE2 plasma levels in cord blood from healthy term neonates using mass spectrometry, and to evaluate the impact of these concentrations on neonatal platelet function.

**Methods**

**Sample collection**

After informed consent 25 self-reported healthy adult volunteers were included into the study. Exclusion criteria were anticoagulant or antiplatelet treatment. Cord blood was obtained from 25 healthy term neonates, following uncomplicated delivery after 39 to 42 weeks of gestation.

**Quantification of prostaglandin E2 plasma levels**

Quantification of PGE2 was done utilising negative ion chemical ionisation (NICI) gas chromatography-mass spectrometry (GC-MS) according to Leis et al. (36).

**Pre-analytics**

Approved by the local ethics committee, venous blood from adult volunteers and cord blood was drawn into S-Monovette® tubes (3 ml) from Sarstedt, previously spiked with 300 µl of 2 % EDTA solution containing 2.1 mM indomethacin. Samples were kept on ice and plasma was obtained within 30 minutes (min) by centrifugation (2500 × g, 4°C, 10 min). One ml 0.02% formic acid was placed into a 10 ml polypropylene tube and 1 ml of the respective plasma sample was added. Fifty µl (1 ng) D4 -PGE2 (Cayman, Ann Arbor, MI, USA) was added before the sample was brought to pH 3.0–3.5 by addition of 1.4 ml 0.5 % formic acid. Samples were stored at –80°C for further processing.

**Extraction procedures**

After thawing 8 ml of ethyl acetate was added to the mixture and prostaglandins were extracted by end-over-end mixing for 10 min. Complete phase separation was obtained by centrifugation (2200 × g, 20°C, 5 min), and the upper phases (ethyl acetate) were transferred into 5 ml glas tubes before evaporation under nitrogen stream at 40°C. Samples were taken up in solvent A (2 ml diethyl ether/petrol ether, 75:25, v/v). 0.5 g Silicar-CC4 (Mallinckrodt, St. Louis, MO; USA) was filled into Poly-Prep Chromatography Columns (Biorad, Munich, Germany) and conditioned with 2 ml of solvent A. Samples were applied to the solid phase and washed twice with 2 ml solvent A. Prostaglandins were eluted with 4 ml of solvent B (ethyl acetate/methanol, 9:1, v/v), and samples were dried under nitrogen stream.

**Derivatisations**

The carboxyl groups of prostaglandins were converted to pentafluorobenzyl esters by treatment with 10 µl of disopropylethylamine and 50 µl of a solution of pentafluorobenzyl bromide in acetonitrile.
trile (7%, w/w) for 10 min at room temperature. The reaction was stopped by evaporation under nitrogen stream.

Keto groups were methoximated by treatment with 50 µl of a solution of methoxime hydrochloride pyridine (2%, w/w) for 2 hours at 75°C. Aqua bidest. (0.4 ml) and N-Hexane (2.5 ml) was added, and samples were extracted by end-over-end mixing for 10 min. Complete phase separation was obtained by centrifugation (2200 × g, 20°C, 5 min), and the upper phases (N-Hexane) were transferred into 5 ml glass tubes before evaporation under nitrogen stream.

The dry residues were treated with 50 µl of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1% Trimethylsilyl chloride in pyridine (2/1, w/w) for 20 min at room temperature, and then again dried under nitrogen stream. The samples were dissolved in 50 µl N-Hexane/1 % N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and transferred into autosampler vials.

GC-MS

Calibration curves were measured by preparing standard dilutions containing 2500 ng, 1250 ml, 625 ng, 312.5 ng, 156.25 ng, and 78.125 ng PGE2 (Cayman). Standards were derivatised as mentioned above, without CC4-extraction.

For sample measurements a Thermo ISQ Single Quadrupole GC-MS (Thermo Fisher Scientific, Rockford, IL, USA) was used. The gas chromatograph was equipped with a Thermo TraceGOLD TG-SQC column (15 m × 0.25 mm). The splitless Grob injector was kept at 280°C. The column was kept at 160°C for 1 min, then programmed to 310°C with an increase of 40°C/min. The column was directly connected to the mass spectrometer. Helium was used as carrier gas. NICI spectra were recorded with an electron energy of 120 eV and an emission current of 0.1 A. Methane was used as moderating gas (1.5 ml/min). Carboxylate anions formed under NICI conditions by loss of the pentafluorobenzyl moiety were monitored by selected ion monitoring at 524 m/z (native PGE2) and 528 m/z (D4-PGE2). Chromatograms were used for quantification by comparing peak areas of the deuterated standard and the native compound.

Platelet aggregation testing

Commercial PGE2 and prostaglandin I2 standards (Cayman) were dissolved in methanol in a concentration of 1 mg/ml and stored in 10 µl aliquots at −80°C. Standards were diluted with isotonic saline prior to measurements. Aggregation measurements were performed on a Multiplate analyser (Multiplate Services GmbH, Munich, Germany) with citrated whole blood. Samples were transferred into measurement cells and incubated with prostaglandin E2 in final concentrations of 1 ng/ml, 10 ng/ml, and 1 µg/ml or with 3 pg/ml prostaglandin I2 for 5 min. Aggregation was triggered by addition of one of the following agonists in final concentrations: ADP (6.5 µM), arachidonic acid (0.5 mM), collagen (3.2 µg/ml), TRAP-6 (32 µM).

Additionally, citrated whole blood was preincubated with 0.5 µM of the EP4 receptor antagonist L-161,982 (Cayman) or the EP3 receptor antagonist L-798,106 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 10 min, respectively, prior to addition of PGE2 and aggregation measurements as described above.

Intracellular cAMP concentrations

Intracellular cAMP concentrations were performed before and after incubation of platelets with PGE2 using a commercially available direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). Briefly, platelet suspensions (2 ml each) were prepared as described above and platelet counts were adjusted to 200 × 10⁹/ml.

![Figure 1: Boxplots of PGE2 concentrations determined by GC-MS in adult (N = 25) and cord blood (N = 25) samples. Whiskers indicate minimum and maximum values, respectively.](image-url)
Samples were incubated with PGE\(_2\) (1 ng/ml or 10 ng/ml final concentration) or prostaglandin I\(_1\) (3 pg/ml final concentration) for 5 min and then pelleted by centrifugation (2500 × g, 4°C, 10 min). Samples were lysed in 500 µl 0.1 M HCl, incubated for 10 min, and residual fragments were sedimented by centrifugation (2500 × g, 4°C, 10 min). Supernatants were transferred into another vial and frozen at –20°C for further processing. After thawing samples were analysed using ELISA according to manufacturer’s instruction.

### Flow cytometric analysis of EP\(_4\) receptor levels

Citrated cord blood or venous adult blood (5 ml) was centrifuged (250 × g, 20°C, 10 min) to obtain platelet-rich plasma. A volume of 250 µl of a 2% EDTA solution was added and platelets were sedimented by centrifugation (1000 × g, 20°C, 15 min). Platelets were washed twice with 5 ml washing buffer, containing 1 mg/ml bovine serum albumin and 0.1 M D(+)-glucose in PBS (pH 7.4). After resuspension in 5 ml washing buffer, the mixture was centrifuged (250 × g, 20°C, 10 min) to remove residual contaminating cells. The supernatant was carefully transferred into another vial and diluted with washing buffer (1:8, v/v).

Aliquots of 250 µl were centrifuged (1000 × g, 20°C, 5 min) and resuspended in 50 µl fixation solution of the FIX&PERM kit (An der Grub, Kaumberg, Austria). After incubation for 15 min at room temperature samples were washed with 250 µl PBS and resuspended in 50 µl permeabilisation reagent of the same kit followed by another incubation step (15 min, room temperature). Samples were washed with 250 µl PBS and blocked by treatment with 50 µl Ultra V Blocking solution (Thermo Fisher Scientific) at 4°C for 30 min. After blocking samples were washed with 250 µl PBS.

The primary monoclonal mouse EP\(_4\) antibody and the isotype control (both from Santa Cruz Biotechnology Inc.) were diluted with antibody diluent (DAKO, Glostrup, Denmark) to a concentration of 20 µg/ml. Two aliquots of each sample were incubated with EP\(_4\) antibody or isotype control, respectively, at 4°C for 30 min. Two control aliquots were incubated with washing buffer.

The secondary Alexa Fluor 647 goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) was diluted with antibody diluent to a concentration of 4 µg/ml.

After another washing step with 250 µl PBS samples and one control were incubated with 50 µl secondary antibody at 4°C for 30 min. The second control was incubated with washing buffer and served as blank. The samples were read on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

### Results

#### Sample information

Cord blood samples were obtained from 13 male neonates with 40.38 ± 1.19 (mean ± SD) gestation weeks and 12 female neonates with 40.42 ± 1.24 (mean ± SD) gestation weeks, all with an one-minute Apgar score of 9. The adult control group was composed of 12 males with 41.08 ± 12.85 (mean ± SD) years of age and 13 females with 43.54 ± 12.96 (mean ± SD) years of age with an overall range of 24 to 63 years.

#### Quantification of prostaglandin E\(_2\) plasma levels

Comparison of calculated peak areas of d\(_1\)-PGE\(_2\) and native PGE\(_2\) showed a linear response with PGE\(_2\) concentration (3 pg/ml final concentration) or prostaglandin I\(_2\) (1 ng/ml or 10 ng/ml final concentration).

#### Solid phase extraction of samples resulted in good detection of PGE\(_2\) levels in adult and cord blood samples. All samples exhibited

### Table 1: Results from whole blood aggregation measurements of adult samples (N = 25) and cord blood samples (N=25) with increasing concentrations of spiked PGE\(_2\). Data is depicted in percentage in comparison to aggregation with vehicle alone. Statistical significance for deviation from vehicle calculated with paired t-test was assumed at *p<0.05 and **p<0.001.

<table>
<thead>
<tr>
<th></th>
<th>1 ng/ml PGE(_2) [% of vehicle]</th>
<th>10 ng/ml PGE(_2) [% of vehicle]</th>
<th>1 µg/ml PGE(_2) [% of vehicle]</th>
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<td></td>
<td>Adult</td>
<td>Cord blood</td>
<td>Adult</td>
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<tr>
<td>ADP</td>
<td>91.2 ± 8.7*</td>
<td>100.8 ± 9.0</td>
<td>84.5 ± 7.6**</td>
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<tr>
<td>Arachidonic acid</td>
<td>95.4 ± 6.5</td>
<td>103.4 ± 10.2</td>
<td>85.2 ± 5.7*</td>
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<tr>
<td>Collagen</td>
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<td>93.7 ± 12.4</td>
<td>89.3 ± 4.9*</td>
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<tr>
<td>TRAP-6</td>
<td>97.9 ± 6.3</td>
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higher levels than the limit of quantification (LOQ: 15 pg/ml). Three adult samples spiked with 1 ng/ml PGE$_2$ showed good recovery rates (97 ± 6%; mean ± SD).

Levels in neonatal cord blood were significantly higher than in adult blood. There was complete separation between the two groups. Quantification of all samples showed a mean PGE$_2$ plasma concentration of 9.526 ± 9.268 ng/ml (mean ± SD) in cord blood vs 0.110 ± 0.107 ng/ml in adults (p<0.001) (Figure 1).

**Flow cytometric analysis of EP$_4$ receptor levels**

Histograms of both, adult and cord blood samples stained for EP$_4$ receptors showed a significant shift to the right when compared with the respective isotype controls, indicating that the receptor is also expressed on neonatal platelets (Figure 2) (p<0.001).

Ratios of geographical means between stained samples and isotype controls were comparable (2.19 ± 0.85 vs 2.43 ± 0.68; mean

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**Figure 3:** Representative whole blood aggregation profiles with increasing concentrations of spiked PGE$_2$ or PGI$_2$. Increase in impedance was transformed to aggregation units (AU). Respective profiles depict a cord blood sample (A) and an adult sample (B) with ADP-induced aggregation, as well as aggregation of the same adult sample induced with arachidonic acid (C), collagen (D), ristocetin (E) and TRAP-6 (F).
± SD), suggesting no significant difference in surface receptor expression between the two groups.

**Platelet aggregation testing**

**Adult samples**

Addition of different concentrations of PGE₂ to adult samples and subsequent whole blood impedance aggregometry resulted in a slight but dose-dependent reduction of platelet aggregation induced by various agonists (▶ Table 1). ADP-induced aggregation was most affected, showing a significant decrease at the lowest spiked concentration, while TRAP-6 induced aggregation did not show a significant reduction even with 1 µg/ml PGE₂ added.

**Cord blood samples**

Cord blood samples exhibited lower whole blood aggregation than adult samples with all agonists. Additional reduction with exogenous PGE₂ was only observed using ADP (▶ Table 1). Baselevel aggregation with this agonist was 336 ± 51 AU*min with cord blood samples and 517 ± 78 AU*min with adult samples (mean ± SD). Despite already high physiological plasma concentrations reduced aggregation was observed when exogenous PGE₂ was added (▶ Figure 3). However, even with the highest concentration of spiked PGE₂ (1 µg/ml) the absolute ADP-induced aggregatory response of adult samples was slightly higher than that of cord blood samples treated with vehicle (389 ± 46 AU*min vs 336 ± 51 AU*min; mean ± SD).

**Effects of EP₃ and EP₄ receptor antagonists**

Adult samples incubated with the EP₃ antagonist prior to inducing aggregation with ADP exhibited more susceptibility to the inhibiting effect of PGE₂ than samples incubated with vehicle (▶ Figure 4). Incubation with the EP₄ receptor antagonist reversed the inhibitory effect of PGE₂ showing a slight but dose-dependent increase of aggregation as long as PGE₂ concentrations did not exceed antagonist concentrations. In contrast to adult samples, preincubation with EP₃ receptor or EP₄ receptor antagonists did not significantly alter the responses to PGE₂ in cord blood samples as compared to vehicle (▶ Figure 5).

**Intracellular cAMP concentrations**

Intracellular cAMP concentration in untreated platelets was determined with ELISA. The mean intracellular cAMP concentration was higher in cord blood samples than in adult samples (12.71 ± 4.79 pg/ml vs 7.72 ± 4.29 pg/ml; mean ± SD; P ± 0.5). Spiking of adult platelets with 1 ng or 10 ng PGE₂ caused a dose-dependent increase in cAMP levels, while neonatal platelets exhibited no change (▶ Table 2). Addition of prostacyclin (PGI₂) resulted in higher cAMP concentrations in both sample groups. However, the relative PGI₂-induced increase was lower in cord blood samples.
Correlations

Correlations between platelet aggregation data and intracellular cAMP concentrations were calculated with Pearson’s r correlation. Significant inverse correlations between aggregation response and intracellular cAMP concentrations were found with collagen- and ADP-induced aggregation in both, adult and cord blood samples (▶Figure 6).

Discussion

We evaluated the impact of PGE₂ from cord blood on platelet function, because high neonatal plasma levels could impair platelet function, thus, explaining some of the findings from in vitro experiments with cord blood (32). On the other hand, a rapid decline after birth would fit the picture of well functioning primary hemostasis in neonates (31).

We wanted to test this hypothesis by quantifying actual PGE₂ plasma levels from umbilical cord blood. PGE₂ levels are known to rise towards end of gestation and peak at accouchement before rapidly declining due to catabolism via pulmonary circulation (28). Therefore, cord blood provides a snapshot of maximum PGE₂ levels in the neonate at birth.

Using GC-MS we found an increase by more than 80-fold in cord blood as compared to venous adult samples. These PGE₂ concentrations were even higher than those reported previously with ELISA measurements in lambs (31). However, our measured cord blood plasma levels (9 ng/ml) were lower than those previously used to demonstrate an activating (35 ng/ml) or inhibitory (211 µg/ml) action of PGE₂ in murine platelets (32). This study was conducted under the premises that the inhibitory effect was mediated by activation of the prostacyclin receptor at high PGE₂ concentrations. Later, the EP4 receptor was identified as primary target for PGE₂-induced inhibitory action in human platelets by three independent research groups (33–35).

To evaluate any effect mediated by EP4 receptors we had to clarify whether they are actually present on the neonatal platelet surface. Using flow cytometry we found an expression of EP4 receptors on neonatal platelets from cord blood that was comparable to adult values. This was in contrast to other G protein-coupled receptors, such as PARs and α2-adrenergic receptors that have been shown to be present at lower levels in neonatal platelets (16, 37). However, a study demonstrating comparable levels of TXA₂ receptors on neonatal and adult platelets shows that this is not a general phenomenon affecting all G protein-coupled receptor types (14). Nevertheless, differences in downstream signalling pathways cannot be excluded.
Our platelet aggregation experiments with adult samples and spiked PGE$_2$ demonstrated a dose dependent inhibitory effect in the concentration range of endogenous cord blood PGE$_2$ levels. The degree of PGE$_2$ inhibition on platelet aggregation varied depending on the agonist. With ADP and arachidonic acid a significant reduction of aggregation was observable at increasing concentrations of PGE$_2$. On the other hand, no significant reduction could be found with collagen at the lowest spiked PGE$_2$ concentration (1 ng/ml), and with TRAP-6 no reduction could be observed at all. Hence, we argue that the influence of PGE$_2$ on platelet aggregation correlates with the agonist's potency. ADP is considered a relatively weak agonist, while collagen and thrombin (accordingly also TRAP-6) are known as strong aggregatory stimuli (38). Response of cord blood samples to exogenous PGE$_2$ was blunted by large amounts of endogenous PGE$_2$ already present in the samples. Hence, it is conceivable that a reduction with spiked PGE$_2$ was only observable in ADP induced aggregation, which was most affected with adult samples too.

Aggregation experiments including preincubation of adult samples with the EP$_3$ receptor antagonist resulted in an increased inhibitory effect of PGE$_2$, while preincubation with the EP$_4$ receptor antagonist led to a slightly activating effect of PGE$_2$. These results are in line with previously published findings arguing for a proaggregatory effect of PGE$_2$ mediated by EP$_3$ receptors, and an anti-aggregatory effect mediated by EP$_4$ receptors (32, 33). Our antagonist studies show that at physiological cord blood PGE$_2$ levels the inhibitory EP$_4$ mediated stimulus dominates over the EP$_3$ receptor mediated activating effect. This is particularly interesting, because PGE$_2$ has been shown to exhibit activating properties at low concentrations and inhibiting properties at high concentrations (32). However, our levels were substantially lower than those previously used for demonstrating the activating effect arguing for alternating dominance of those two stimuli over the whole PGE$_2$ concentration range.

Using a commercial ELISA we observed that basal levels of intracellular cAMP are higher in platelets from cord blood than in adult platelets, which could partially explain the reported hyporesponsiveness of neonatal platelets to various agonists. Spiking of platelet suspensions with additional PGE$_2$ resulted in an increase
of intracellular cAMP in adult platelets, but no significant changes were observed in platelets from cord blood.

We believe that the influence that PGE$_2$ can have on the intracellular cAMP concentration in adult platelets is maxed out at physiological neonatal levels. This is in line with our finding that adult cAMP concentrations were comparable with neonatal basal levels after addition of 10 ng/ml PGE$_2$, but could be further increased by addition of PGI$_2$. Compared to PGI$_2$, PGE$_2$ could only induce a moderate cAMP increase even in adult platelets. This is entirely conceivable, because PGI$_2$ is known to completely abrogate platelet aggregation, which was not observable with PGE$_2$.

However, we believe that the EP$_4$ receptor mediated inhibitory effect is not solely caused by an increase of intracellular cAMP. This notion is supported by data of Philipose et al. demonstrating that pretreatment of platelets with an adenylyl cyclase inhibitor was unable to prevent the inhibitory effect of an EP$_4$ agonist on platelet aggregation (33).

The collective data from experiments with cord blood samples suggests that most EP$_4$ receptors on neonatal platelets have already been activated by physiologically high levels of PGE$_2$. This is in line with increased cAMP levels and observable reduced platelet aggregation. Subsequent receptor desensitisation of PGE$_2$ receptors could explain the blunted responses to exogenous PGE$_2$ of platelets from cord blood in platelet aggregation and intracellular cAMP accumulation, as well as the lack of effect when cord blood samples were incubated with EP$_3$ or EP$_4$ receptor antagonists.

We found a correlation between cAMP base levels and data from ADP- or collagen-aggregation with adult and cord blood samples. Given that these were only statistically weak correlations, we cannot exclude a potential correlation with other agonists at higher case numbers.

However, we found no direct correlation between PGE$_2$ plasma levels and intracellular cAMP concentrations, and no correlation between PGE$_2$ plasma levels and platelet aggregation. Although we saw an effect on intracellular cAMP concentrations and platelet aggregation when exogenous PGE$_2$ was added, there are several other factors affecting one of those two parameters in correlation analyses, potentially obscuring the effect of endogenous PGE$_2$.

Experiments demonstrating the impact of exogenous PGE$_2$ at cord blood levels enable the differential evaluation of varying PGE$_2$ concentrations on platelet function, and can only be performed with adult samples that contain minimal amounts of endogenous PGE$_2$. Cord blood samples are not feasible for this procedure, because they already contain large amounts of endogenous PGE$_2$. Nevertheless, we performed the same experiments also with cord blood samples demonstrating desensitisation to PGE$_2$. Given that EP$_4$ receptor levels are comparable in both sample groups, we argue that the effect of endogenous PGE$_2$ of cord blood on neonatal platelets and that of exogenous PGE$_2$ in the same concentration on adult platelets is comparable.

Plasma levels of PGE$_2$ are not commonly quantified with mass spectrometry, but with ELISA, most likely due to ease-of-use. Also with adult samples GC-MS might not be the best option as plasma levels are usually in the low pg/ml range scratching the LOQ of some instruments. However, for the determination of plasma levels in cord blood samples there are several advantages over ELISAs. All commonly used ELISAs exhibit 70% cross-reactivity with PGE$_1$, which, in our case, might have resulted in completely different results. Our GC-MS featured a high specificity for PGE$_2$ due to a different retention time than PGE$_1$. Also, this methodology enables high-throughput measurements of larger sample numbers, allowing future clinical studies to evaluate PGE$_2$ plasma levels as a potential predictor for patent ductus arteriosus. The high inter-individual variation of PGE$_2$ plasma levels in cord blood samples most likely reflects actual conditions in vivo, because these levels change at a very rapid rate. Hence, the variation can be attributed to different gestation length and several factors during accouchement. Nevertheless, all cord blood samples exhibited substantially higher levels than adult samples.

A limitation of this study might be the surrogate nature of PGE$_2$ plasma levels from cord blood for actual plasma levels in the neonate at birth, as blood was only available several minutes after accouchement. However, studies with neonates are always problematic due to difficulties in obtaining proper blood samples in ample amounts. Obtaining larger amounts of platelets from neonatal venous blood is hardly ethically justified. PGE$_2$ levels did not correlate with the time-interval between accouchement and blood collection. Also, preliminary experiments showed that PGE$_2$ levels remained stable over more than one hour, when stored in tubes without indomethacin (data not shown).

## Conclusion

Our data demonstrate very high PGE$_2$ plasma levels in cord blood that inhibit platelet function. This inhibitory effect is due to predominance of EP$_4$ receptor-mediated signalling. Inhibition upon

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**What is known about this topic?**

- Neonatal platelets exhibit hypoaggregability *in vitro*, but support thrombin generation as well as adult platelets, and contribute to occlusion of the ductus arteriosus. Neonates are not prone to easy bruising.
- Prostaglandin E$_4$ promotes or inhibits reactivity of murine and adult human platelets concentration-dependently via EP$_3$ and EP$_4$ receptors.
- High plasma levels of immunoreactive prostaglandin E$_2$ have been demonstrated in lambs at birth followed by a steep decline after accouchement.

**What does this paper add?**

- Plasma levels of prostaglandin E$_2$ were quantified in human adult and cord blood samples for the first time using mass spectrometry.
- Neonatal and adult platelets exhibit comparable levels of EP$_4$ receptors.
- Physiological plasma levels of prostaglandin E$_2$ in neonates at birth increase intracellular cAMP and inhibit platelet function due to dominance of EP$_4$ receptor-mediated signalling.
spiking with exogenous PGE₂ is more pronounced in adult platelets than neonatal platelets, due to desensitisation by endogenous PGE₂ in cord blood. It is strongly dependent on the type of agonist and probably constitutes only one of many factors responsible for the hyporesponsiveness of neonatal platelets. Nevertheless, peak levels of PGE₂ at accouchement may provide an additional inhibiting stimulus potentially protecting against birth stress-induced platelet activation.

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Conflicts of interest

None declared.

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