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Decreased Platelet Counts and Serum Levels of VEGF-A, PDGF-BB, and BDNF in Extremely Preterm Infants Developing Severe ROP

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Keywords
Retinopathy of prematurity · Platelets · Vascular endothelial growth factor · Platelet-derived growth factor · Brain-derived neurotrophic factor

Abstract
Introduction: Thrombocytopenia has been identified as an independent risk factor for retinopathy of prematurity (ROP), although underlying mechanisms are unknown. In this study, the association of platelet count and serum platelet-derived factors with ROP was investigated. ***Methods:*** Data for 78 infants born at gestational age (GA) <28 weeks were included. Infants were classified as having no/mild ROP or severe ROP. Serum levels of vascular endothelial growth factor A, platelet-derived growth factor BB, and brain-derived neurotrophic factor were measured in serum samples collected from birth until postmenstrual age (PMA) 40 weeks. Platelet counts were obtained from samples taken for clinical indication. ***Results:*** Postnatal platelet counts and serum concentrations of the 3 growth factors followed the same postnatal pattern, with lower levels in infants developing severe ROP at PMA 32 and 36 weeks ($p < 0.05$–0.001). With adjustment for GA, low platelet counts and low serum concentrations of all 3 factors at PMA 32 weeks were significantly associated with severe ROP. Serum concentrations of all 3 factors also strongly correlated with platelet count ($p < 0.001$). ***Conclusion:*** In this article, we show that ROP, platelet counts, and specific pro-angiogenic factors correlate. These data suggest that platelet-released factors might be involved in the regulation of retinal and systemic angiogenesis after extremely preterm birth. Further investigations are needed.

Introduction

Infants born extremely preterm are immature and at high risk of developing both acute and long-lasting morbidities, including retinopathy of prematurity (ROP). In extremely preterm new-born infants, the retina is not ful-

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ly vascularized. At birth, a rapid change from the in utero milieu to the relatively hyperoxic ex utero milieu takes place. In the hyperoxic postnatal environment, local expression of oxygen-regulated pro-angiogenic factors decreases, and blood vessel development is suppressed in the first phase of ROP. With maturation of the neural retina, oxygen and nutrition demands increase, followed by enhanced expression of pro-angiogenic factors, including vascular endothelial growth factor A (VEGF-A). If ROP develops, this period constitutes the second phase of the disease, starting at approximately postmenstrual age (PMA) 30 weeks [1]. In this critical transition period, physiological vessel development or development of ROP occurs without or with pathological neovascularization. The exact mechanisms regulating retinal vascularization are not known.

Severe ROP is characterized by pathological retinal neovascularization that is visible several weeks to months after birth. Treatment usually begins at around 12 weeks postnatal age (PNA) or 36 weeks PMA. Immaturity, low birth weight, oxygen supplementation, and fluctuating oxygenation are well-established risk factors for the development of ROP, but several other risk factors have been described (reviewed in [2]). In recent years, thrombocytopenia has been identified as an independent risk factor for severe ROP [3–9], but the associated mechanisms are not understood. Proteomic studies have identified release of several pro-angiogenic, anti-angiogenic, and neurogenic factors in response to platelet activation, including VEGF, platelet-derived growth factor (PDGF), and brain-derived neurotrophic factor (BDNF) [10]. Some studies have suggested that ligand binding to endothelial cells followed by secretion of α-granule contents activates different signaling pathways involved in blood vessel growth and maturation. With this process, platelets might deposit angiogenic and neurogenic regulatory proteins in a localized manner [11, 12], but data are inconclusive [10]. Experimentally, factors released from activated platelets promote cell migration and endothelial cell tube formation [13, 14].

Regulation of vessel formation involves a complex interplay among different cell types. VEGF is a key player in this process. In rodents, VEGF protects from hyperoxia-induced vessel loss, and intraocular administration of anti-VEGF drugs suppresses hypoxia-induced neovascularization [15]. In the presence of a VEGF receptor antagonist, a compensatory angiogenic effect of platelet releasate has been observed in vitro [16].

A complex cross-talk among different cell types also regulates vascular maturation, involving endothelial, smooth muscle, retinal ganglion, and Müller cells, along with astrocytes and pericytes. Platelet-derived growth factor BB (PDGF-BB) has been described as a connecting factor between endothelial cells and retinal ganglion cells, astrocytes, and Müller cells during angiogenesis, and as a key factor for pericyte recruitment [17]. In mice, administration of PDGF-BB affects retinal vascular remodeling in a time-dependent manner through different developmental stages [18]. Only pericytes engaged in ongoing cell migration respond to exogenous PDGF-BB, suggesting a shift in responsiveness upon maturation. In mice, antagonism of both PDGF-BB and VEGF-A has an additive suppressive effect on pathological neovascularization. The period of VEGF dependence corresponds to the period before new vessels acquire a pericyte coating [18, 19].

The expression of BDNF in ganglion cells, astrocytes, and Müller cells was described in the 1990s. More recently, BDNF expression has also been reported in endothelial cells [20]. We and others have found lower levels of circulating BDNF in blood samples from infants with ROP compared to infants without ROP [21–23]. In addition, although genetic variants in the BDNF gene have been associated with severe ROP [24, 25], data are inconclusive [26].

Among extremely preterm infants, thrombocytopenia is one of the most common hematologic conditions in the neonatal period. Its prevalence is inversely correlated with gestational age (GA) at birth [27]. We speculate that thrombocytopenia is a risk factor for ROP, associated at least partly with decreased levels of locally released platelet-derived angiogenic and neurotrophic factors because of a low platelet count. Most platelet-derived factors are expressed in a diversity of cells, and the origin of measured concentrations in the circulation is diverse. However, levels of serum VEGF-A and BDNF are highly correlated with platelet count, supporting the hypothesis that these values in serum originate to a high degree from released α-granule content [28–30]. Furthermore, serum concentrations of VEGF increase with clotting time [31], and BDNF concentrations are low in platelet-poor plasma, whereas concentrations are approximately 100–200-fold higher in pooled serum than in plasma because of release during the in vitro clotting process. Given this pattern, serum levels could be used as an indirect measurement of platelet-derived levels of these factors. The aim of this study was to investigate the associations between plasma platelet count and serum levels of selected platelet-derived factors with severe ROP in a cohort of extremely preterm infants.

Table 1. Clinical characteristics

	No ROP $n = 17$	Mild ROP (stage 1 and 2) $n = 30$	Severe ROP (stage 3 or treated) $n = 31$	All $N = 78$
GA at birth, mean (SD), wk	26.1 (1.4)	25.4 (1.3)	24.3 (1.1)	25.1 (1.4)
Birth weight, mean (SD), g	1,024 (194)	776 (199)	693 (170)	797 (223)
Weight SD score, mean (SD)	−0.10 (0.87)	−1.20 (1.27)	−0.85 (1.24)	−0.82 (1.20)
Male, *n* (%)	13 (76)	11 (37)	19 (61)	43 (55)
Bronchopulmonary dysplasia, *n* (%)	4 (24)	15 (50)	20 (65)	39 (50)
Necrotizing enterocolitis	0 (0)	1 (3.3)	4 (13)	5 (6.4)
Patent ductus arteriosus	6 (35)	29 (97)	26 (81)	54 (69)
Sepsis, *n* (%)	3 (18)	12 (40)	15 (48)	30 (39)
Thrombocytopenia, $<100 \times 10^9$/L, *n* (%)	2 (12)	13 (43)	23 (74)	38 (49)
Platelet transfusion	0 (0)	6 (20)	18 (58)	24 (31)

GA, gestational age; ROP, retinopathy of prematurity; SD, standard deviation.

Methods

Study Design

The study was performed within the Donna Mega Study, a randomized, open-label, controlled trial conducted at Queen Silvia Children's Hospital in Gothenburg, Sweden (NCT 02760472). Briefly, infants born at <28 weeks GA were randomly allocated to parenteral lipid emulsion with SMOFlipid® or with Clinoleic®. Exclusion criteria were major congenital malformations. The primary outcome was ROP, as described by Najm et al. [32]. Secondary analyses included evaluation of postnatal angiogenic and neurotrophic biomarkers in relation to ROP. The study design has been described previously [32].

Study Population

From April 2013 to September 2015, parents of 90 of 138 eligible infants agreed to participate in the study. Of these, data for 78 infants (43 male; 55%, mean [SD] GA, 25.5 [1.4] weeks) with a known ROP outcome were evaluated in the final analyses. Laser therapy was used for all infants treated for ROP in this study.

Eye Examinations

ROP screening started at PNA 5–6 weeks but not before PMA 31 weeks. Retinal examinations through dilated pupils were performed at a rate of twice weekly to every 2 weeks, depending on ROP severity, until the retina was fully vascularized or the condition was considered stable. ROP was classified based on the international classification [33], and the recommendations of the Early Treatment for Retinopathy of Prematurity Cooperative Group [34] were followed for treatment. The study outcome of severe ROP was defined as stage 3 and/or treated ROP.

Morbidities

Diagnoses, including bronchopulmonary dysplasia, necrotizing enterocolitis, persistent arteriosus, sepsis, and thrombocytopenia, were retrieved from clinical records. Bronchopulmonary dysplasia was defined as moderate-to-severe lung disease with a need for oxygen supplementation at PMA 36 weeks, necrotizing enterocolitis was diagnosed by clinical signs and radiologic findings (Bell's stages 2–3), and persistent arteriosus was registered when the infant had clinical symptoms that required either pharmacological or surgical treatment. Sepsis was diagnosed by clinical symptoms accompanied by a positive blood culture. When the culture contained *Staphylococcus epidermidis*, an elevated C-reactive protein (>20 mg/L) was required for diagnosis. National recommendations defined neonatal thrombocytopenia as a platelet count $<100 \times 10^9$/L.

Blood Sampling and Laboratory Analyses

Serum samples were collected at birth (cord blood); at postnatal days 1, 7, 14, and 28; and at PMA 32, 36, and 40 weeks. Samples were centrifuged, aliquoted, and placed at −70°C within 2 h of being taken. VEGF-A, BDNF, and PDGF-BB were analyzed using the ELLA multi-analyte platform (Biotechne, Minneapolis, MN, USA) according to the protocol provided by the manufacturer. Briefly, 50 µL sample, diluted 1:4 in SD13 buffer, was placed in cartridge wells, and then the atomized analysis procedure was finalized within 70 min. Raw data were processed using Simple Plex Explorer Software (Biotechne). Inter-assay coefficients of variation (CVs) tested among 40 assays were 7.00, 7.09, and 7.76% at 35, 475, and 1,667 pg/mL, respectively, for VEGF-A; 7.05, 6.83, and 5.01% at 220, 7,402, and 10,271 pg/mL, respectively, for BDNF; and 4.51, 3.60, and 4.69% at 7.5, 332, and 945 pg/mL, respectively, for PDGF-BB. Intra-assay CVs based on 9 measurements of a pooled serum sample were 4.77% at 502 pg/mL for VEGF-A, 5.51% at 7,731 pg/mL for BDNF, and 5.24% at 983 pg/mL for PDGF-BB.

Plasma samples for platelet counts were collected for clinical indications and analyzed by Advia 2120i (Siemens Healthcare GmbH, Erlangen, Germany) and a modified method in Technicon H6000. The same methods were used at all hospitals in the Västra Götaland region where infants were hospitalized. For each infant, mean platelet count per day was performed and a mean weekly platelet count calculated.

Statistics

Mean and SD are presented for normally distributed continuous variables and median and range for skewed continuous variables. The Mann-Whitney U test was used for comparisons be-

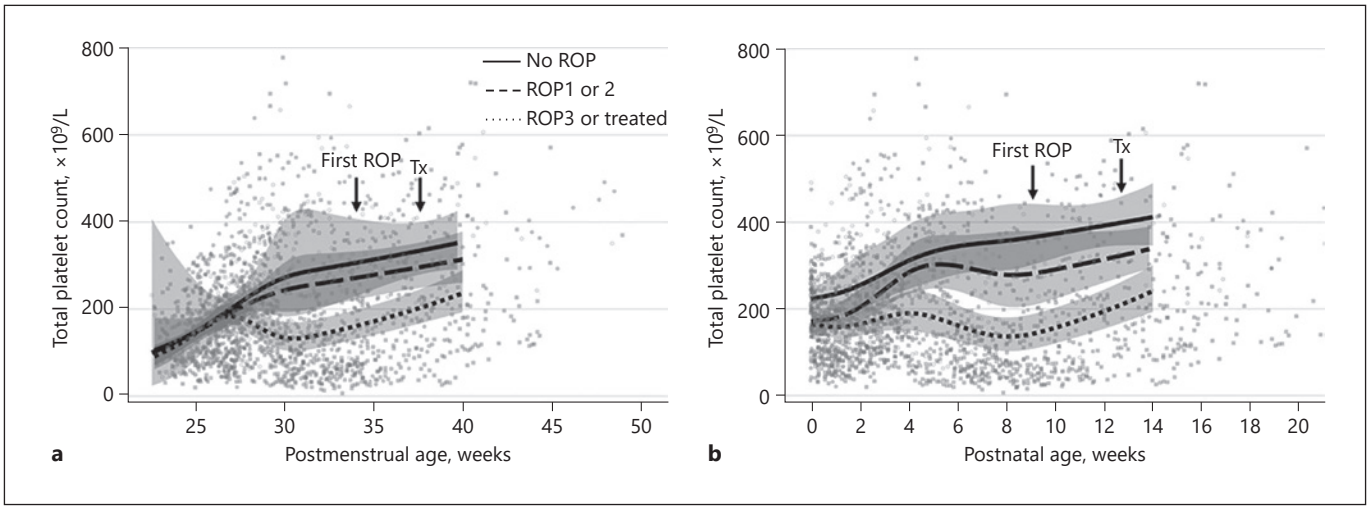

Fig. 1. Platelet counts, with means and 95% CIs shown for infants without ROP or with mild ROP (stage 1 or 2) or severe ROP (stage 3, untreated and treated), respectively. Indicated with arrows are mean age when ROP was first detected and mean age for treatment (Tx). **a** Values corresponding to PMA. **b** Values corresponding to postnatal age. CI, confidence interval; ROP, retinopathy of prematurity; PMA, postmenstrual age.

tween 2 independent groups. For descriptive purposes, random coefficient models were applied when estimating mean and 95% confidence interval (CI) for platelet count, VEGF-A, BDNF, and PDGF-BB for no, mild, and severe ROP handling of the underlying timescales, PMA and PNA in separate analyses, using natural cubic splines. Residual plots were reviewed and robust sandwich estimators used to adjust for heteroscedasticity. Univariable and GA-adjusted logistic regression analyses were performed for evaluation of the association of VEGF-A, BDNF, and PDGF-BB with severe ROP. ORs with 95% CIs, and c-statistics with 5% CIs are presented. A model with a c-statistic >0.70 was considered acceptable. Missing data were not imputed.

Relationships between platelet count and VEGF-A, BDNF, or PDGF-BB were investigated using Spearman correlations. All statistical analyses were performed using SPSS 23 for Microsoft Windows (IBM, Armonk, NY, USA) and SAS Software version 9.4 (SAS Institute Inc., Cary, NC, USA), with two-tailed tests and a p value <0.05 considered as significant.

Results

Clinical Characteristics

The clinical characteristics of this study population have been described previously [32, 35]. Table 1 shows the clinical characteristics for this cohort, stratified into "no ROP" (n = 17), "mild ROP" (stage 1 or 2; n = 30), and "severe ROP" (stage 3 or 4, untreated or treated; n = 31).

Lower Platelet Count in Infants with Proliferative ROP

Platelet count in relation to ROP was investigated by weekly mean platelet count in samples taken for clinical indications, according to both PMA and PNA. As shown in Figure 1, the numerical differences in platelet counts between infants without ROP and with mild ROP were quite small. However, infants with severe ROP had a lower platelet count from PMA 30 weeks (shown in Fig. 1a) and PNA 5 weeks (shown in Fig. 1b). For statistical analyses, infants were grouped as non/mild ROP (non-ROP, n = 17; ROP stage 1, n = 8; ROP stage 2, n = 22) or severe ROP (n = 23). Mann-Whitney U results confirmed lower platelet counts in infants with severe ROP than in infants with no or mild ROP at PMA 32 and 36 weeks (shown in Table 2). Regarding PNA, sufficient data for statistical analyses were available only for 4 weeks after birth; however, the temporal pattern according to PNA followed the same pattern as for PMA.

Logistic regression was used for further analysis of the association between low platelet count and ROP. Platelet count was investigated per 50 units by univariate logistic regression analyses, and in a second step adjusted for GA at birth. In univariate analysis, low platelet count was identified as a predictor for severe ROP at PMA 32 (OR 0.70 [95% CI 0.56–0.87] per 50-unit increase; p = 0.002) and 36 weeks (OR 0.61 [95% CI 0.43–0.87] per 50-unit increase; p = 0.021). After adjustment for GA, however, only low platelet count at PMA 36 weeks remained significant (OR 0.65 [95% CI 0.45–0.94]; p = 0.021) among the 33 infants whose data were available (shown in Table 3). The c-statistics for the univariable model with total platelet count at PMA 36 weeks PMA was high (0.82; 95% CI 0.66–0.98).

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Table 2. Platelet count and serum levels of VEGF-A, PDGF-BB, and BDNF according to PMA stratified by ROP severity

PMA, wk	No/mild ROP TPC × 10^9	Severe ROP TPC × 10^9	*p* value	No/mild ROP VEGF-A	Severe ROP VEGF-A	*p* value	No/mild ROP PDGF-BB	Severe ROP PDGF-BB	*p* value	No/mild ROP BDNF	Severe ROP BDNF	*p* value
24	171 (85; 249) *n* = 9	160 (53.0; 390) *n* = 15	0.72	668 (352; 1,033) *n* = 8	447 (100; 1,893) *n* = 16	0.34	535 (287; 2,603) *n* = 8	478 (19; 3,069) *n* = 16	0.74	2,781 (721; 17,014) *n* = 8	3,631 (60; 8,486) *n* = 16	0.56
25	223 (41; 319) *n* = 14	137 (68; 320) *n* = 23	0.015	526 (126; 1,657) *n* = 16	536 (69; 1,879) *n* = 24	0.69	666 (36; 2,153) *n* = 16	472 (119; 2,072) *n* = 24	0.52	3,582 (348; 11,748) *n* = 16	2,329 (312; 16,067) *n* = 24	0.79
26	188 (45; 413) *n* = 26	195 (56; 300) *n* = 22	0.99	462 (108; 2,199) *n* = 27	444 (111; 1,580) *n* = 23	0.29	653 (28; 2,361) *n* = 27	672 (97; 3,128) *n* = 23	0.79	3,574 (117; 15,601) *n* = 27	2,125 (334; 19,703) *n* = 23	0.38
27	198 (21; 418) *n* = 33	153 (56; 478) *n* = 19	0.089	346.6 (68; 1,245) *n* = 36	660 (137; 1,258) *n* = 18	0.22	627 (43; 1,615) *n* = 35	685 (85; 2,986) *n* = 18	0.62	3,197 (214; 10,455) *n* = 36	4,548 (449; 15,990) *n* = 18	0.56
28	216 (71; 536) *n* = 31	228 (56; 511) *n* = 13	1.00	790 (133; 1,845) *n* = 31	843 (117; 1,233) *n* = 14	0.72	927 (215; 2,982) *n* = 31	977 (37; 4,970) *n* = 14	0.78	7,188 (694; 17,978) *n* = 31	8,417 (150; 23,901) *n* = 14	0.93
29	316 (132; 500) *n* = 19	282 (69; 556) *n* = 7	0.89	862 (117; 2,212) *n* = 22	775 (125; 2,062) *n* = 10	0.48	1,388 (61; 3,339) *n* = 22	1,318 (221; 3,475) *n* = 10	0.95	7,631 (405; 20,815) *n* = 22	7,594 (1,365; 18,524) *n* = 10	0.76
32	289 (126; 570) *n* = 27	117 (14; 553) *n* = 26	<0.001	805 (130; 2,310) *n* = 41	336.8 (731; 941) *n* = 29	<0.0001	1,219 (242; 2,392) *n* = 41	468 (7; 2,236) *n* = 29	<0.001	10,425 (835; 24,061) *n* = 41	3,455 (54; 18,917) *n* = 29	<0.001
36	328 (55; 478) *n* = 15	161.5 (30; 514) *n* = 18	0.0018	556 (81; 1,678) *n* = 34	398.3 (123; 1,119) *n* = 25	0.020	1,057 (81; 2,138) *n* = 34	772 (46; 2,495) *n* = 25	0.0089	14,326 (593; 31,155) *n* = 34	7,210 (248; 28,758) *n* = 25	0.0021
40	333 (138; 607) *n* = 12	235 (108; 485) *n* = 13	0.17	546 (68; 1,306) *n* = 33	394.6 (93; 1,022) *n* = 19	0.28	1,395 (391; 2,592) *n* = 33	1,297 (204; 2,703) *n* = 19	0.25	19,422 (1,696; 37,547) *n* = 33	13,007 (1,773; 25,523) *n* = 19	0.032

Median and range (min–max) were presented for continuous variables. Mild ROP: ROP stage 1 or 2. Severe ROP: ROP stage 3 and/or treated ROP. BDNF, brain-derived neurotrophic factor; PDGF-BB, platelet-derived growth factor BB; PMA, postmenstrual age; ROP, retinopathy of prematurity; TPC, total platelet count; VEGF-A, vascular endothelial growth factor A; wk, weeks.

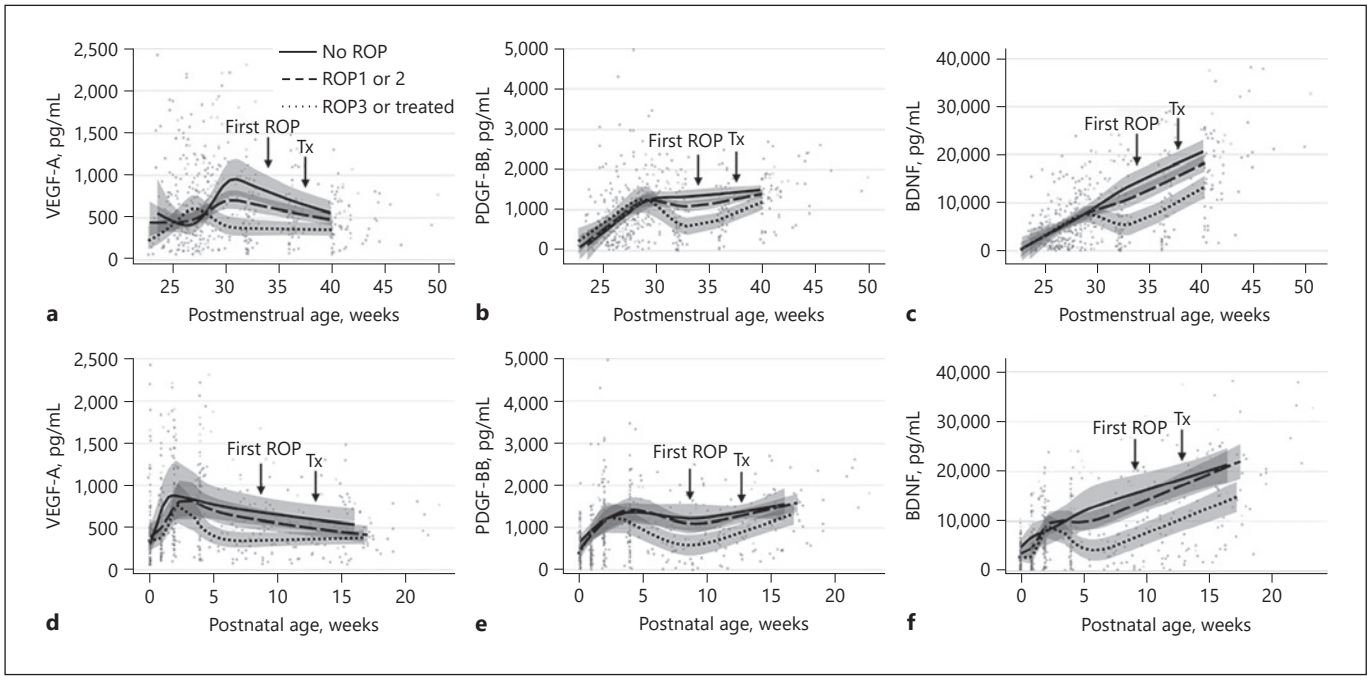

Fig. 2. Serum concentrations of VEGF-A (**a**, **d**), PDGF-BB (**b**, **e**), and BDNF (**c**, **f**), with means and 95% CIs shown for infants with no ROP or with mild ROP (stage 1 or 2) or severe ROP (stage 3, untreated and treated), respectively. Arrows indicate mean age when ROP was first detected and mean age for treatment (Tx). Upper panel shows values corresponding to PMA and lower panel shows values corresponding to postnatal age. CI, confidence interval; ROP, retinopathy of prematurity; VEGF-A, vascular endothelial growth factor A; PDGF-BB, platelet-derived growth factor BB; BDNF, brain-derived neurotrophic factor; PMA, postmenstrual age.

BDNF, VEGF-A, and PDGF-BB Associated with Severe ROP in the Second Phase of ROP

The longitudinal pattern for VEGF-A, PDGF-BB, and BDNF in infants with no, mild, or severe ROP was similar to the pattern shown for platelet count, with lower levels from PMA approximately 30 weeks (shown in Fig. 2a–c) or PNA approximately 5 weeks (shown in Fig. 2d–f) in infants developing severe ROP compared to infants with no or mild ROP. According to PMA, descriptive statistics revealed lower VEGF-A, PDGF-BB, and BDNF levels in infants developing severe ROP compared to those with no or mild ROP (shown in Table 2). By PNA, enough data for statistical analyses were available only to PNA 4 weeks. During the first weeks of life, serum concentrations of PDGF-BB and VEGF-A were similar regardless of later ROP. However, BDNF levels were lower at PNA 1 and 4 weeks in infants developing severe ROP compared to levels in infants with no or mild ROP. Median BDNF (min; max) at PNA 1 week were 2,035 (60; 15,232) pg/mL in infants developing severe ROP, compared to 4,264 (257; 17,978) pg/mL in infants with no or mild ROP ($p = 0.038$). At PNA 4 weeks, corresponding levels of BDNF were 5,122 (270; 18,524) pg/mL in infants developing severe ROP compared to 9,626 (405; 24,154) pg/mL in infants with no or mild ROP ($p = 0.003$).

According to PMA, univariable logistic regression analyses identified low levels of VEGF-A, PDGF-BB, and BDNF at PMA 32 and 36 weeks and BDNF at PMA 40 weeks as associated with severe ROP. After adjustment for GA, low serum levels of VEGF-A, PDGF-BB, and BDNF at PMA 32 weeks remained significant. All 3 variables at PMA 32 weeks showed high predictive ability when analyzed alone, as well, with c-statistics from 0.80 to 0.85 (details shown in Table 3).

Correlation of BDNF, VEGF, and PDGF with Platelet Count

We used correlation analyses to investigate the relationship between platelet count and serum levels of VEGF-A, PDGF-BB, and BDNF. Only platelet values obtained from samples taken the same day as VEGF-A, PDGF-BB, and BDNF were included. Results showed a moderate to strong correlation between platelet count and VEGF-A, PDGF-BB, and BDNF, respectively (r =

Table 3. Analysis of VEGF-A, PDGF-BB, and BDNF serum levels as risk factors for severe ROP using logistic regression, before and after adjustment for GA at birth

Variables	Univariable			Adjusted for GA	
	OR (95% CI) severe ROP	*p* value	c-statistics (95% CI)	OR (95% CI) severe ROP	*p* value
TPC at PMA 32 weeks (OR per 50×10^9/L) *n* = 53	0.70 (0.56–0.87)	0.002	0.82 (0.69–0.94)	0.79 (0.62–1.02)	0.067
TPC at PMA 36 weeks (OR per 50×10^9/L) *n* = 33	0.61 (0.43–0.87)	0.006	0.82 (0.66–0.98)	0.65 (0.45–0.94)	0.021
VEGF-A at PMA 32 weeks (OR per 100 pg/mL), *n* = 70	0.64 (0.51–0.80)	<0.001	0.85 (0.76–0.94)	0.68 (0.54–0.85)	<0.001
VEGF-A at PMA 36 weeks (OR per 100 pg/mL), *n* = 59	0.81 (0.67–0.98)	0.029	0.68 (0.54–0.82)	0.87 (0.70–1.06)	0.17
VEGF-A at PMA 40 weeks (OR per 100 pg/mL), *n* = 52	0.87 (0.70–1.08)	0.21	0.59 (0.43–0.76)	0.88 (0.68–1.14)	0.33
PDGF-BB at PMA 32 weeks (OR per 100 pg/mL), *n* = 70	0.79 (0.70–0.89)	<0.001	0.83 (0.72–0.94)	0.84 (0.75–0.95)	0.005
PDGF-BB at PMA 36 weeks (OR per 100 pg/mL), *n* = 59	0.89 (0.79–0.99)	0.030	0.70 (0.56–0.85)	0.92 (0.82–1.03)	0.16
PDGF-BB at PMA 40 weeks (OR per 100 pg/mL), *n* = 52	0.94 (0.84–1.04)	0.22	0.60 (0.42–0.77)	0.94 (0.83–1.06)	0.29
BDNF at PMA 32 weeks (OR per 1,000 pg/mL), *n* = 70	0.82 (0.73–0.92)	<0.001	0.80 (0.69–0.91)	0.87 (0.78–0.98)	0.021
BDNF at PMA 36 weeks (OR per 1,000 pg/mL), *n* = 59	0.89 (0.82–0.97)	0.005	0.74 (0.60–0.87)	0.93 (0.85–1.01)	0.077
BDNF at PMA 40 weeks (OR per 1,000 pg/mL), *n* = 52	0.91 (0.84–0.99)	0.025	0.68 (0.53–0.84)	0.92 (0.84–1.01)	0.089

Severe ROP: ROP stage 3 and/or treated ROP. BDNF, brain-derived neurotrophic factor; GA, gestational age; OR, odds ratio; CI, confidence interval; PDGF-BB, platelet-derived growth factor BB; PMA, postmenstrual age; ROP, retinopathy of prematurity; TPC, total platelet count; VEGF-A, vascular endothelial growth factor A; wk, weeks.

0.42, $r = 0.66$ and $r = 0.70$, respectively; $p < 0.001$) (shown in Fig. 3). In addition, pairwise correlation analyses for VEGF-A, BDNF, and PDGF-BB revealed moderate-to-strong correlations among the factors (VEGF-A vs. PDGF-BB, $r_s = 0.65$; VEGF-A vs. BDNF, $r_s = 0.52$; and BDNF vs. PDGF-BB, $r_s = 0.78$; $p < 0.001$).

Discussion

The current results represent the first findings of a strong correlation between low platelet count and low serum levels of VEGF-A, PDGF-BB, and BDNF, from PMA of approximately 30 weeks and PNA of 5 weeks in infants developing severe ROP. Although platelets are carriers of a number of pro- and anti-angiogenic factors, and low platelet count or thrombocytopenia is an independent risk factor for ROP [3, 4, 7, 9, 36, 37], the specific factors behind the association have been unclear.

From around PMA 30 weeks, during the second phase of ROP, the peripheral retina becomes hypoxic. VEGF is upregulated, and some infants develop proliferative ROP, which is treated with the aim of reducing retinal VEGF. It was during this phase that we found an association of low platelet counts and low levels of circulating growth factors with severe ROP. These results are not associated with external supply of platelets by transfusion. Infants developing severe ROP receive platelet transfusion most frequently. In addition, most platelet transfusions are given earlier than the PMA 30 weeks. Platelets are carriers of both pro- and anti-angiogenic factors and might function to prevent retinal neovascularization. In a mouse model of ROP, platelet depletion increased neovascularization, whereas platelet transfusion at postnatal day (P)15 and P16 suppressed neovascularization. These effects were mediated by factors released from platelet granules [36]. In these animals, retinal VEGF mRNA and protein expression increased upon platelet depletion and decreased after transfusion, supporting the hypothesis of a local anti-angiogenic effect of platelets.

In the late 1960s and early 1970s, Gimbron et al. [40] and others reported that platelets not only regulate hemostasis but also influence new blood vessel development and that thrombocytopenia leads to elevated vascular permeability [38–40]. Furthermore, platelet activity changes through developmental stages. Neonatal platelets have been characterized as hyporeactive compared to adult platelets and also compared to platelets from full-term infants in some studies (reviewed in [41]).

Platelet α-granules contain high amounts of both pro- and anti-angiogenic factors, but it is generally accepted that activation of platelets has an overall stimulatory net effect on angiogenesis. In vitro, releasate from activated platelets induces tube formation and cell migration of human umbilical cord endothelial vein cells [14]. Heat treatment negatively affects cell proliferation, suggesting that at least some activation factors are proteins.

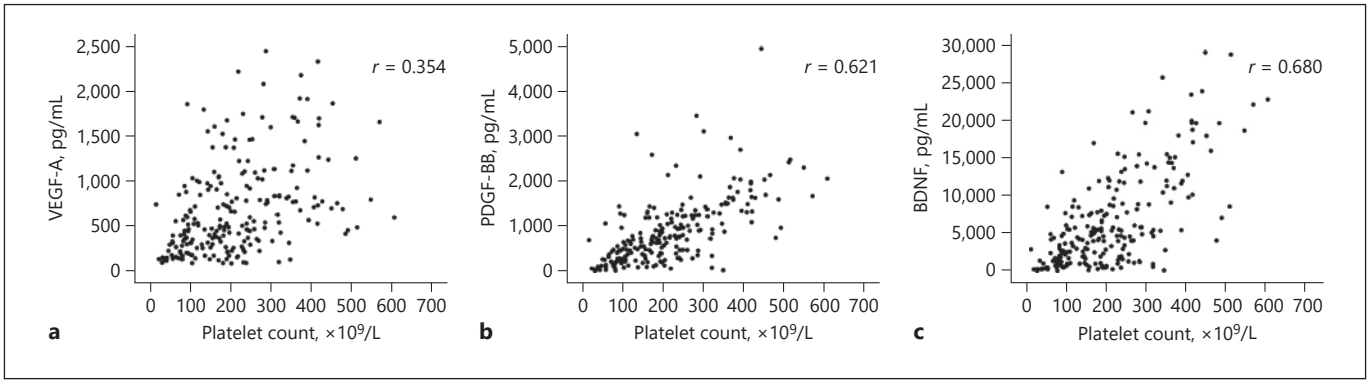

Fig. 3. Correlation of platelet counts with VEGF-A (**a**), PDGF-BB (**b**), and BDNF (**c**). VEGF-A, vascular endothelial growth factor A; PDGF-BB, platelet-derived growth factor BB; BDNF, brain-derived neurotrophic factor.

After preterm birth, physiological retinal vascularization probably depends on timing and the availability of pro- and anti-angiogenic factors. This idea is supported by findings in neonates with and without ROP and in rats of different ratios of pro-angiogenic VEGF/anti-angiogenic pigment epithelial-derived factor [42, 43].

In this study, the temporal patterns of the pro-angiogenic factors VEGF-A and PDGF-BB, as well as the neurotrophic factor BDNF, were evaluated in relation to platelet count and to ROP. It has previously been shown that high levels of these factors are stored in platelet α-granules and released as a response to activation. Levels measured in serum mainly originate from platelet release in response to in vitro coagulation [28, 31, 44, 45]. These previous findings together with the strong correlation identified in the present study of VEGF-A, PDGF-BB, and BDNF in serum with platelet counts in plasma suggest that serum levels of at least these factors mainly are carried by platelets and released during clotting. The role of VEGF-A in the development of ROP is well established, and PDGF-BB and BDNF also have been associated with ROP [18, 19, 21–24, 46, 47].

Data are inconclusive regarding systemic levels of VEGF in relation to ROP [48]. In the current work, VEGF-A levels during the first postnatal weeks were similar and independent of later ROP. From a PMA of approximately 30 weeks, infants developing severe ROP had lower VEGF-A than infants with no or mild ROP. These results contrast with our previous data showing increased circulating VEGF levels prior to the first signs of ROP [49] but are in line with other studies demonstrating the same postnatal pattern. The reasons for the contrasting results are not clear. However, in our previous study, weekly samples were collected from birth until PMA 40 weeks, whereas in the present study, samples were collected at birth, 1, 2, and 4 weeks after birth, and then with a lower resolution at PMA 32, 36, and 40 weeks. Variations in the temporal pattern thus could have been missed. Furthermore, systemic VEGF levels are highly variable within and among individuals, and small sample numbers might have contributed to the divergent results between the studies. Today it is not known how, or if, these factors are influenced by ROP treatment. This could be further elucidated in a follow-up study where samples are collected with higher resolution around the time when proliferative ROP is detected and around the time for treatment.

Our findings do not offer mechanistic explanations for what underlies thrombocytopenia as a risk factor for ROP. This is a limitation and requires investigation. Of interest, in vitro, selective α-granule release depending on stimulatory agents has been reported. For example, thromboxane A2 and adenosine diphosphate stimulation result in opposite effects on VEGF release, cell migration, and tube formation [50]. In vivo, selective release of pro- or anti-angiogenic factors might be involved in the regulation of the local availability of these factors, and in this way, the regulation of vessel development.

In summary, results from this study show a strong correlation of the postnatal pattern of platelet count with serum levels of the neurogenic and angiogenic factors VEGF-A, PDGF-BB, and BDNF. Low platelet count during the second phase of ROP was associated with low levels of these factors. Although increased retinal levels of VEGF during the hypoxic second phase of ROP cause uncontrolled neovascularization that needs treatment to lower retinal VEGF concentrations, we found decreased serum VEGF concentrations during this same period.

Taking our clinical findings together with previously published experimental findings, we suggest that depending on the physiological condition, platelet-released factor might be involved in the regulation of retinal angiogenesis after extremely preterm birth. Experimental studies are needed to further elucidate mechanisms underlying thrombocytopenia as a risk factor for ROP.

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Statement of Ethics

The study was performed according to the World Medical Association Declaration of Helsinki, and the trial was approved by the Regional Ethical Board, Gothenburg (Dnr 303-11), at the University of Gothenburg. Informed written consent was obtained for all participants from their parents or guardians. No manufacturer of the parenteral products contributed to the design of the study, accrual or analysis of the data, or preparation of the manuscript.

Conflict of Interest Statement

The authors declare no conflicts of interest to declare.

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Author Contributions

G.H., P.L. and A.H. contributed to the conception of the research; all authors contributed to the design of the research; G.H., P.L., and A.P. contributed to the acquisition and analysis of the data; all authors contributed to the interpretation of the data; and G.H. drafted the manuscript. All authors critically revised the manuscript, and approved the final manuscript.

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