Changes in thrombopoiesis and platelet reactivity in extremely low birth weight infants undergoing erythropoietin therapy for treatment of anaemia of prematurity

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Summary

Erythropoietin (Epo) is frequently administered to premature infants to stimulate erythropoiesis. There is evidence from studies in animals and healthy adults that Epo also interacts with thrombopoiesis and platelet function. This study investigates the effect of Epo therapy on platelet reactivity, peripheral platelet counts and thiazole orange-positive (TO+) platelets in extremely low birth weight (ELBW) infants. In a randomised-controlled trial, ELBW infants with a birth weight ≤ 800g and a gestational age ≤ 32 weeks were either randomised to a group receiving Epo during the first weeks of life or to a control group. Our results show that thrombin receptor-activating peptide (TRAP-6) -induced expression of P-selectin increased significantly during the first two weeks of Epo treatment. With the exception of week five, the number of TO+ platelets was significantly higher during the first eight weeks in Epo-treated infants compared to controls. The increase of TO+ platelets was not paralleled by an increase in total platelet count. We can conclude that Epo therapy has a short-lasting effect on platelet reactivity to TRAP-6 in ELBW infants during the first two weeks of life. Furthermore, Epo therapy is associated with an increase in the number of TO+ platelets compared to controls.

Keywords

Erythropoietin, platelet reactivity, VLBW infants, TRAP, TO+ platelets

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Introduction

Premature infants, especially very low birth weight (VLBW [birth weight < 1500 g]) infants, frequently suffer from anaemia of prematurity, and associated problems such as increased susceptibility to infections, a need for increased oxygen or insufficient weight gain during the first weeks of life. The anaemia can in part be explained by the iatrogenic blood loss, the poor iron stores and the low serum erythropoietin (Epo) levels of VLBW infants compared to similarly anaemic older children or adults (1–3). For treatment of anaemia and prevention of associated complications, premature infants often receive multiple blood transfusions during their stay in the neonatal intensive care unit. Although the safety of blood transfusions has improved, a residual risk for complications like transfusion-associated viral transmission, fluid imbalances, haemolysis and iron overload remains (4–6). Therapy with recombinant Epo has become an alternative to multiple blood transfusions and a number of controlled trials on its application in VLBW infants have been published so far (2, 7–13). The results are in part conflicting with regard to reduction of blood transfusions and optimal treatment strategies (14). Variables like birth weight, gestational age (GA), dosage of Epo and iron and the initiation and duration of therapy likely account for the heterogenous results. Especially in the subgroup of critically ill extremely low birth weight (ELBW [birth weight < 1000 g]) infants, considerable iatrogenic blood loss during the first weeks

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of life limits a significant impact on transfusion requirements (15). In spite of the heterogeneity of the studies, the efficacy of Epo therapy on stimulation of erythropoiesis in VLBW infants is proven (2, 8, 9, 16–18). As a consequence of enhanced erythropoiesis, reticulocyte counts and haematocrit significantly increase. Furthermore, Epo therapy decreases the number of blood transfusions and the total blood volume infused, in particular in premature infants with a birth weight between 800 and 1300 g.

Besides the proliferative effect on erythroid progenitor cells, there is evidence from animal studies (19) and *in vivo* studies in humans (20), that Epo therapy enhances platelet reactivity as measured by an increase in P-selectin-positive (P-selectin+) platelets. Furthermore, a transient stimulating effect on thrombopoiesis was demonstrated by an increase in the platelet count of healthy young men (20).

Platelets of premature and term infants are considered to be dysfunctional compared to adults'. These platelets are markedly hyporeactive to a number of agonists such as thrombin and ADP (21–24). The reasons for this hyporeactivity are not yet fully understood. Authors have attributed this observation to a post-receptor defect in a signal transduction pathway (23).

We hypothesised that Epo may also affect thrombopoiesis and reverse or ameliorate platelet dysfunction in premature infants. Accordingly, the aim of the current study was to evaluate the effect of Epo therapy on platelet reactivity and thrombopoiesis in ELBW infants.

Materials and methods

Study design and population

The study design was an open-label randomised controlled trial. Premature infants with a birth weight ≤800 g and a GA ≤32 weeks admitted to the neonatal intensive care units of the Department of Paediatrics, University of Vienna, Austria, were eligible for inclusion in the study. Exclusion criteria were haemolytic disease, maternal causes of neonatal thrombocytopenia, major haemorrhage (defined as intraventricular haemorrhage III° or IV°, or gastrointestinal or pulmonary haemorrhage associated with a haemoglobin drop of > 2 g/dL in 24 h), twin-to-twin transfusion syndrome, major congenital malformations and known gastrointestinal abnormalities. Infants were assigned randomly to Epo therapy or to a control group. Randomisation assignment was performed using sealed opaque envelopes. The study was approved by the Ethics Committee of the University of Vienna. Written consent was given by the parents after full explanation of the procedure.

Study protocol

For better understanding of the study setting, application and administration of study medication in Epo and control group are listed in table 1 (Erythropoietin: Erypo®; Janssen-Cilag Pharma, Vienna, Austria; Iron dextran: INFeD®; Schein Pharmaceutical Inc., NJ, USA; Iron polymaltose complex: Maltofer®; Vifor International, St. Gallen, Switzerland; Folic acid: Folsan®; Solvay Pharma, Klosterneuburg, Austria; Vitamin B_{12} :Vitamin B_{12} Lannacher®; Lannacher Heilmittel, Lannach, Austria) (12, 15, 25). All study medication was administered up to 40 weeks of GA corrected for prematurity or until discharge. Iron dextran and Vitamin B_{12} (when applied intravenously) were added to the parenteral nutrition. Once the infant was on full enteral feedings, Vitamin B_{12} was applied together with Epo in the same syringe.

As Epo has been shown to be safe at lower doses, there has recently been a tendency to increase the dose of Epo in order to increase efficacy. We therefore decided to apply a consistently higher dose (i.v. and s.c.) during the whole study period. Initial doses were based on birth weight and were adjusted to the current weight twice a week. Criteria for withholding the study drug (Epo, iron) included neutropenia (neutrophil count below 500 /μL), and severe sepsis (defined as culture proven sepsis requiring catecholamine or ventilatory support). The study drug was restarted when neutrophil counts increased over 500 /μL again. In case of severe sepsis, Epo therapy was interrupted for three days and then restarted. Parenteral iron was not administered while Epo was held. During the study, a strict transfusion protocol as published by Shannon et al. was abided (2). If the transfusion criteria were fulfilled, a daily maximum of 15 mL/kg of packed red blood cells (irradiated, washed, and filtered) was transfused until the infant's haematocrit rose ≥ 40 %. Exact volume of blood transfused, number of donors and increase in haematocrit and haemoglobin were recorded on the case report form.

Sampling and analysis of primary outcome variables

In both groups, full blood count and differential, reticulocyte count, thiazole orange positive (TO+) platelet count and platelet reactivity were measured weekly in samples obtained from venipuncture (usually from a hand or foot vein) or from a central venous line. Baseline values for these parameters were obtained from cord blood except TO+ platelet count and platelet reactivity. To avoid preanalytical platelet activation, blood for platelet

Table 1: Therapy protocol. All study medication was administered up to 40 weeks of GA corrected for prematurity or until discharge.

	Application	Epo group	Control Group
Erythropoietin	intravenously †	300 U/kg/d	-
Erythropoietin	subcutaneously ‡	700 U/kg, 3 x/week	-
Iron dextran	intravenously †	1.5 mg/kg/d	-
Iron polymaltose complex	orally	9 mg/kg/d ‡‡	9 mg/kg/d ††
Folic acid	orally	100 μg/kg/d ††	60 μg/kg/d ††
Vitamin B_{12}	intravenously †	3 μg/kg/d	-
Vitamin B_{12}	subcutaneously	21 μg/kg/week	-
Vitamin E	orally	5 mg/d	5 mg/d

† Intravenously as long as intravenous access was available
‡ Subcoutaneously after achievement of full enteral feedings
†† Orally from 15th day of life on or when infant tolerated 60 mL/kg of enteral feeding, whichever came first
‡‡ Orally when infant tolerated 60 mL/kg of enteral feeding

reactivity and reticulated platelets analysis was always taken from a freshly pricked venipuncture.

Complete blood counts with differentials and reticulocyte counts were performed using an automatic blood cell counter (Cell Dyn 4000®; Abbott, Vienna, Austria).

Measurement of platelet reactivity with thrombin receptor-activating peptide

Platelet reactivity was measured by quantifying % of P-selectin+ platelets after stimulation with a platelet agonist. For this purpose, 50 µL of venous whole blood were collected into plastic tubes containing 6µL of 129mM trisodium citrate. The sample was then mixed gently and 20 µL of the sample were diluted with 200 µL of phosphate-buffered saline (PBS). Twenty µL of this dilution were incubated with 10 µL of a Phycoerythrin (PE)-labelled P-selectin (CD62) antibody (Immunotech, Marseille, France) and PAR1 thrombin receptor (SFLLRN) -activating peptide dilution at 23°C for 15 min. Three concentrations of TRAP (TRAP [TRAP-6®, Bachem, Switzerland; molecular weight 748.9 g/mol]) were used: 3.7 µM, 7.4 µM and 14.8 µM. After incubation, 500µL of PBS was added and acquisition on a flow cytometer (FACSCalibur®; Becton Dickinson, San Jose, CA) was started immediately. For the TRAP dilution, 5 mg were dissolved in 1.5 mL of bidestilled water. Ten µL of this solution were further diluted with 2 mL of PBS. The TRAP concentrations were selected according to the concentrations used in experiments investigating platelet reactivity of healthy adults undergoing Epo therapy (20). Given that platelets of neonates are hyporeactive, we presumed that a higher concentration may be needed for sufficient stimulation of platelets and chose an additional third concentration. Results were expressed as percentages of P-selectin+ platelets.

Quantification of thiazole orange-positive platelets

The percentage of TO+ platelets was measured to determine whether an increased platelet reactivity could be due to newly synthesized hyperreactive platelets. Five µL of the citrated blood sample was added to 1mL of paraformaldehyde 1% in PBS and analysed as described previously (26).

Table 2: Patient demographics. P-values were calculated with Mann-Whitney U test. None of the listed parameters showed any statistical significance.

	Epo group (n=21)	Control group (n=19)	p
Gestational age (weeks)	25+4 (23+6-31+2)	25+0 (23+4-28+2)	n.s.
Birth weight (g)	690 (500-800)	690 (467-783)	n.s.
Duration of study period (days)	59 (9-124)	55 (16-130)	n.s.
Hospital stay (days)	97 (59-162)	89 (77-157)	n.s.
Number of transfusions (n)	2 (0-15)	4.5 (0-12)	n.s.
Number of donors (n)	1 (0-10)	3 (0-5)	n.s.
Amount of blood transfused (mL)	28 (0-229)	75 (0-387)	n.s.
Phlebotomy loos (mL)	34 (14-94)	35 (14-94)	n.s.

n.s. Not signiticant

Statistical analysis

Based on studies investigating platelet reactivity in healthy adults undergoing Epo therapy (27), a sample size of 40 was calculated to detect 20% difference in platelet reactivity between the groups, with a power of 80% and a significance level of 0.05.

Results are expressed as the median with upper and lower quartiles or as median with range in the figures and tables, respectively. Given the non-normal distribution of the data, all comparisons were performed using non-parametrical tests. The primary objective was to compare treatment effects between study groups, for which the Mann-Whitney U test was used. A secondary comparison of differences before/after treatment was performed using the Wilcoxon test within the two study groups. The chi-square test was used to test for differences in clinical characteristics between the groups. A p-value < 0.05 was considered statistically significant.

Results

Study population

During the study period between October 2000 and November 2002, 47 infants were eligible for enrolment in the study. Four infants were excluded because of parental refusal (n=2) or intraventricular haemorrhage IV° (n=2), respectively. Three infants died before randomisation. Therefore, the final cohort included 40 infants. Twenty-one and 19 infants were randomised to the intervention group and control group, respectively. Demographic data and clinical characteristics including outcome of both groups are given in Table 2 and 3. No differences were noted for GA, gender, birth weight, duration of study period, hospital stay, weight at discharge or complications of prematurity between the groups. Administration of Epo and Vitamin B_{12} was switched from intravenous to subcutaneous route after a median of 6 weeks (median 43 days, range 19–304). Before starting the clinical trial, we performed *in vitro* experiments mixing Epo and vitamin B12 in a syringe. No precipitation of protein was observed.

Reticulocyte counts and transfusion requirements

There was a considerable reticulocytosis during Epo treatment from week 1 to 10 with an average increase of reticulocyte counts three-fold above the control group. The number of premature infants requiring no transfusion was higher in the Epo group (n=8; 38%) compared to the control group (n=1; 5%) ($p < 0.05$). There was a trend towards a lower cumulative volume of transfused blood (Table 2). Details on the effect of combined therapy of Epo, iron, folic acid and Vit B_{12} on transfusion requirements and red blood cell parameters in ELBW infants will be published separately.

Platelet reactivity in response to thrombin receptor-activating peptide

Both study groups exhibited similar basal P-selectin expression (Fig. 1). During the first two weeks, platelets of Epo-treated infants were significantly more reactive to lower concentrations (3.7 and 7.4 µM) of TRAP than those of the control group (Fig.

1B and 1C). A similar, albeit non-significant, trend towards increased platelet reactivity was observed in the Epo group after stimulation with 14.8 µM TRAP (Fig. 1D). However, this effect was transient and after the third week of therapy the % of P-selectin+ platelets were indistinguishable from those of the control group.

Platelet counts and thiazole orange-positive platelets

No differences in platelet counts were found between the groups (Fig. 2). TO+ platelets increased significantly in the first week and remained high until week 8 of therapy compared to the controls with the exception of week 5 (Fig. 3). There was a significant increase in TO+ platelets compared to baseline in the Epo group. No significant differences for any other parameter were observed in before/after treatment comparison.

Discussion

We hypothesised that Epo therapy could stimulate platelet reactivity and thrombopoiesis in ELBW infants. Epo treatment tran-

Table 3: Clinical characteristics of the study population. P-values were calculated with chi2-test. None of the listed parameters showed any statistical significance.

	Epo group (n=21)	Control group (n=19)	p
Male gender	57% (n=12)	37% (n=7)	n.s.
Death	14% (n=3)	21% (n=4)	n.s.
Persistent ductus arteriosus	14% (n=3)	11% (n=2)	n.s.
Necrotizing enterocolitis	14% (n=3)	0%	n.s.
Periventricular leukomalacia	19% (n=4)	16% (n=3)	n.s.
IVH I and II	33% (n=7)	26% (n=5)	n.s.
IVH III and IV	0%	10% (n=2)	n.s.
ROP ! and II	5% (n=1)	11% (n=2)	n.s.
ROP III and IV	5% (n=1)	0%	n.s.
Bronchopulmonary dysplasia	29% (n=6)	42% (n=8)	n.s.

IVH: Intraventricular haemorrhage; ROP: Retinopathy of prematurity; n.s.: Not significant.

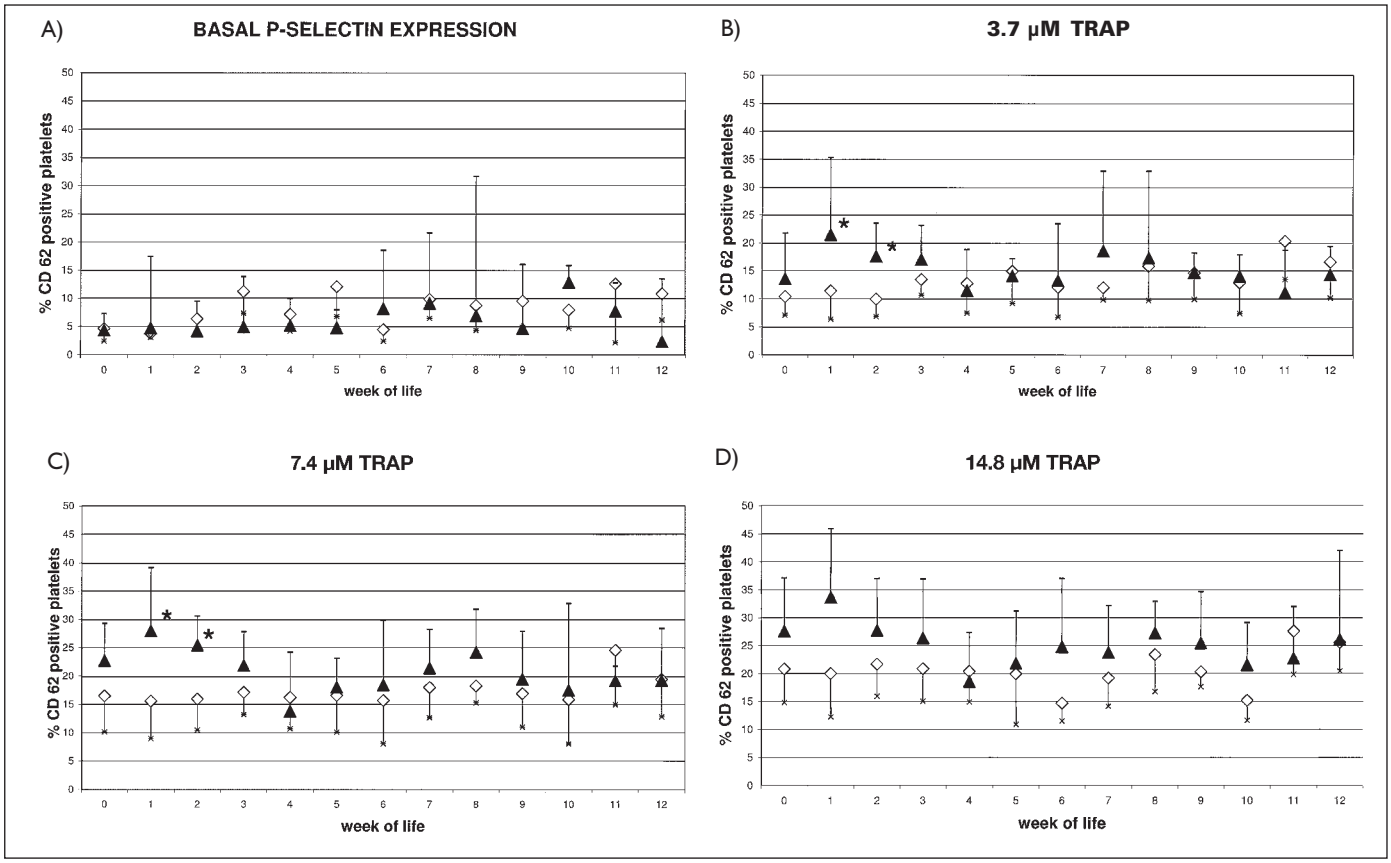

Figure 1: A. Basal P-selectin expression in premature infants either receiving Epo (data presented in median = black triangles, upper quartiles = dashes) or no drug (data presented in median = open diamonds, lower quartiles = crosses). No statistically significant difference was observed between the groups. B-D. Time-dependent effects of Epo treatment on platelet reactivity in whole blood as measured by P-selectin (CD 62)+ platelets in response to three different doses of thrombin receptor-activating peptide (TRAP) (3.7, 7.4 and 14.8 µM). Epo-treated premature infants (data presented as median = black triangles, upper quartiles = dashes) showed a significantly higher P-selectin expression to 3.7 and 7.4 µM TRAP ($p < 0.05$) during the first two therapy weeks in comparison to controls receiving no medication (data presented in median = open diamonds, lower quartiles = crosses). Concomitantly, a similar trend towards increased platelet reactivity in the Epo group after stimulation with the highest used concentration of TRAP (14.8 µM) was observed, but these results did not reach statistical significance.

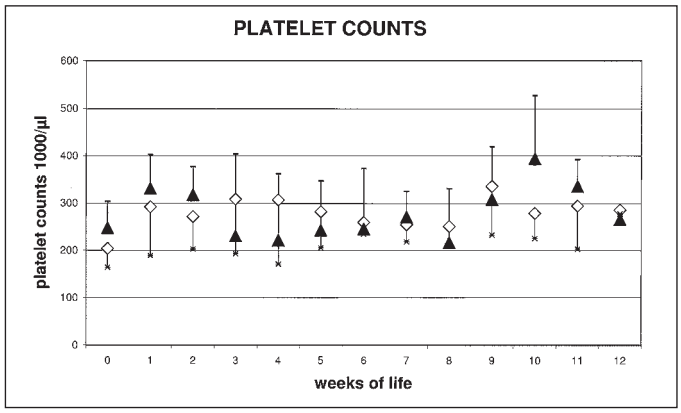

Figure 2: Effects of Epo treatment on platelet counts in premature infants. No differences were observed between the Epo group (data presented as median = black trinangles, upper quartiles = dashes) and controls (data presented as median = open diamonds, lower quartiles = crosses).

siently enhanced platelet reactivity which may potentially indicate an improved platelet function.

TRAP stimulates platelets via the PAR-1 receptor and induces aggregation and surface expression of platelet activation markers including P-selectin (28). Hyporeactivity of platelets is a well-known condition in newborns (21, 23, 24, 29–31). Pietrucha et al. using a similar experimental setting like in the current study, stimulated platelets of term infants with TRAP at a concentration of 8 µM and found approximately 70% P-selectin+ platelets (24). In the present study, platelet reactivity in the control group was only about 15% after stimulation with a similar dosage of TRAP (7.4 µM). Platelet hyporeactivity may depend on GA because a number of platelet agonists have a weaker response in premature infants as compared to term infants (21, 22). However, it is uncertain up to which GA platelets remain hyporeactive compared to adults. While the current study did not include a control group of adults, comparison to our previous studies (20, 32) suggest, that platelets of ELBW infants remain hyporeactive to stimulation with TRAP at least until term age. A recent study found a reduced platelet activation response in children that lasts until adolescence (33).

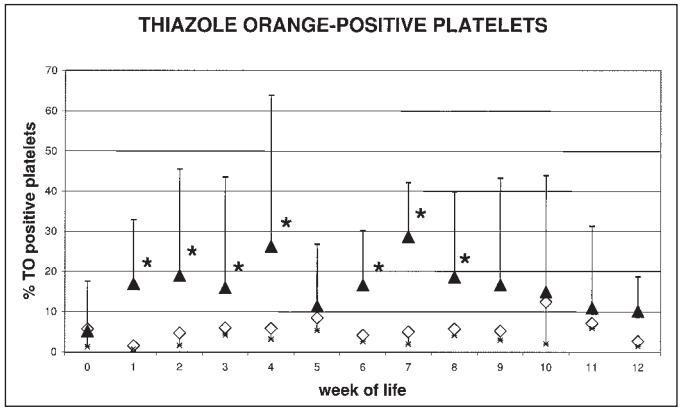

Figure 3: Effects of Epo on the percentage of thiazole orange-positive platelets (reticulated platelets) in premature infants during Epo therapy (data presented as median = black triangles, upper quartiles = dashes). The percentage of thiazole orange-positive platelets started to increase significantly in the first week of therapy and remained high until the eighth week of therapy in comparison to controls (data presented as median = open diamonds, lower quartiles = crosses; $p < 0.05$; except week five p = n.s.).

Epo enhances platelet reactivity in animals and humans (19, 20). In healthy young men, Epo (100 U/kg thrice a week) over a time period of 12 days resulted in an increase of platelet reactivity to TRAP: (20) The percentage of P-selectin+ platelets increased from 20% before treatment to 60% after treatment at concentrations of 3.7 µM TRAP. In ELBW infants, the Epo dose (300 U/kg/d) was even higher but less effective: P-selectin+ platelets increased from 14% to 18% (3.7 µM TRAP) and from 23% to 26% (7.4 µM TRAP), respectively. Hence, compared to adults or term infants (20, 24), platelets of ELBW infants appear to have a reduced functional reserve capacity with a hyporeactive response to platelet agonists such as TRAP. However, our results show that Epo therapy is associated with an increased platelet reactivity in ELBW infants during the first two weeks of therapy.

The current study showed an Epo-associated increase in the synthesis of TO+ platelets which was consistently three- to five-fold higher in Epo-treated infants than in controls (except week 5; Fig. 3). The increase in TO+ platelets lasted the whole study period, while platelet reactivity returned to baseline values in the 3rd week of therapy. Despite an increase in TO+ platelets, platelet counts were not altered in response to Epo therapy (Fig. 2). These results are in agreement with findings from a study on fetuses undergoing cordocentesis between the 21st and 37th week of gestation (27). In this recent report, we found highly elevated numbers of TO+ platelets but normal platelet counts in fetuses as compared to their mothers. These findings may indicate a higher turnover due to a shorter life span of fetal platelets (similar to fetal erythrocytes) or a larger demand of platelets to maintain endothelial integrity.

Alternatively, the high proportion of TO+ platelets found in the present study may be due to non-specific labelling with dye (34). At least in mice, dense granules are lost during platelet aging and that even those platelets have a relatively short half life as compared to the whole circulating platelet population (34). However, we have previously demonstrated that in fetuses full degranulation of platelets with 80 µM TRAP did not reduce TO+ platelets in fetal or maternal blood (27).

Healthy newborn infants were reported to have a smaller proportion of TO+ platelets compared to adults (35). In contrast TO+ platelets counts were increased in premature infants with a GA < 30 weeks compared to term and preterm infants > 30 weeks GA. (36). Platelet counts and percentages of TO+ platelets in the latter study are in good agreement with values measured in our control group (Fig. 3). Finally, fetuses have very high numbers of TO+ platelets (27).

Reports on the effect of Epo on platelet counts in premature infants are controversial. Ohls et al. observed transient decreases in platelet counts during weeks 1, 3 and 8 in infants less than 1000 g, and during weeks 3 to 5 in infants 1001 to 1250 g compared to non-treated controls (15). The current study found no significant differences in the platelet counts between the two study groups (Fig. 2). In contrast, another study reported an Epo-

associated thrombocytosis (platelet counts > $500x10^9$ /mL) in 31% of 114 premature infants (11). Differences between the patient population and co-morbidities may account for the heterogenous findings.

In summary, Epo therapy transiently increases platelet reactivity in ELBW infants during the first two weeks of life. Platelet hyporeactivity as measured by TRAP responsiveness does not normalise in ELBW infants during the first twelve weeks of life. Furthermore, Epo therapy increased TO+ platelets during the whole study period while total platelet counts remain unchanged. The Epo-induced increase in TRAP induced P-selectin expression is markedly less in ELBW infants than adults. Further, this increase in platelet reactivity is transient and unlikely sufficient to provide beneficial effects per se. However, the persistent increase in the number of TO+ platelets could reflect a platelet subpopulation with a different haemostatic profile. This observation deserves further investigations.

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