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NEONTAL THROMBOCYTOPENIA – FROM BLEEDING RISK TO NEW THERAPIES ON THE HORIZON

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Neonatal Thrombocytopenia – From Bleeding Risk to New Therapies on the Horizon

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"To do successful research, you don't need to know everything, you just need to know of one thing that isn't known."

Arthur Schawlow, Nobel prize winner physicist

ABSTRACT

Background: Thrombocytopenia is common in the Neonatal Intensive Care Unit and if severe, it is treated with platelet transfusions to prevent bleeding. Previous studies found a weak correlation between thrombocytopenia and incidence of bleeding. The poor predictive value of the platelet count has highlighted the need for better tests to assess bleeding risk and to guide transfusion decisions. Romiplostim (ROM) and eltrombopag (ELT) – two thrombopoietin (TPO) mimetics approved for treatment of thrombocytopenia in adults – may benefit thrombocytopenic neonates by potentially reducing platelet transfusions.

Objectives: First, the relationship between platelet count and *in vitro* bleeding time (PFA-100 closure times, CTs) in thrombocytopenic neonates was evaluated. Second, other risk factors associated with CTs were determined. Third, the relationship between the PFA-100 CTs and clinical bleeding was assessed. Fourth, *in vitro* responses of human neonatal vs. adult megakaryocyte (MK) progenitors to TPO, ROM and ELT, were compared.

Methods: We conducted a single institution cross-sectional study (*Study 1)*, and a multicenter prospective longitudinal study (*Study 2)*. Blood samples from thrombocytopenic neonates were tested with PFA-100 to measure *in vitro* bleeding time. In addition to platelet counts, other variables including severity of illness were obtained. Bleedings were quantified using the Neonatal Bleeding Assessment Tool (NeoBAT). In a pre-clinical study (*Study 3*), hematopoietic progenitor (CD34+) cells isolated from umbilical cord blood (CB) and adult peripheral blood (PB) were cultured in escalating concentrations of TPO, ROM, or ELT. After 14 days, the number of MKs and their maturation (CD42b expression) and ploidy were evaluated by flow cytometry.

Results: In *Study 1*, we found that all infants with platelet counts >90x10^9/L had normal CT-ADPs. At platelet counts <90x10^9/L, 74 % (14/19) of the infants had normal or minimally prolonged CT-ADPs, while 16 % (3/19) had markedly prolonged values. *Study 2* showed that in preterm infants with gestational age <27 weeks and platelet counts <100×10^9/L, CT-ADP but not platelet counts was associated with NeoBAT scores. This association was robust also after adjustment for demographic and clinical variables. *Study 3* found that with escalating concentrations of TPO, ROM, or ELT, CB progenitors generated 10 times more MKs than PB-MKs. At low concentrations, ELT stimulated megakaryopoiesis, but at high concentrations, ELT suppressed MK differentiation and proliferation via its intracellular iron chelating properties.

Conclusions: The CT-ADP test (an *in vitro* test of whole blood primary hemostasis) is a significantly better marker of bleeding risk than platelet counts among thrombocytopenic preterm neonates. TPO mimetics increased the numbers of cord blood MKs but did not influence their maturation or ploidy level. ELT generated fewer MKs than TPO or ROM. At low concentrations ELT stimulated megakaryopoiesis, but higher concentrations resulted in reduced MK differentiation and proliferation. These findings may have implications for the management of thrombocytopenic infants.

LIST OF SCIENTIFIC PAPERS

I. **Primary Hemostasis in Neonates with Thrombocytopenia.**
Deschmann E, Sola-Visner M, Saxonhouse MA
J Pediatr. 2014 Jan;164(1):167-72. doi: 10.1016/j.jpeds.2013.08.037. Epub 2013 Oct 3.

II. **Association Between In Vitro Bleeding Time and Bleeding in Preterm Infants with Thrombocytopenia.**
Deschmann E, Saxonhouse MA, Feldman HA, Norman M, Barbian M, Sola-Visner M
JAMA Pediatrics Published online February 25, 2019

III. **Bleeding Risk Prediction and Effect of Transfusions in Thrombocytopenic Preterm Neonates.**
Deschmann E, Saxonhouse MA, Feldman HA, Norman M, Barbian M, Sola-Visner M
Submitted

IV. **Iron Status/Eltrombopag Dose Interactions Determine the Response of Cord Blood Megakaryocyte Progenitors to Eltrombopag in Vitro.**
Deschmann E, Liu ZJ, Ramsey H, Feldman HA, Psaila B, Cooper N, Vlachodimitropoulou E, Porter J, Bussel J, Georgieff M, Sola-Visner M
Submitted

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LIST OF ABBREVIATIONS

BT	Bleeding Time
CT	Closure Time
ELT	Eltrombopag
IVH	Intraventricular Hemorrhage
MK	Megakaryocyte
NEC	Necrotizing Enterocolitis
NeoBAT	Neonatal Bleeding Assessment Tool
Neo-HAT Study	Neonatal Hemorrhagic Risk Assessment in Thrombocytopenia Study
NICU	Neonatal Intensive Care Unit
PAR	Protease-Activated Receptor
PFA-100	Platelet Function Analyzer-100
ROM	Romiplostim
TPO	Thrombopoietin
TRALI	Transfusion Associated Lung Injury
VLBW	Very Low Birth Weight

1 INTRODUCTION

Platelets are small cells in the blood that are involved in forming blood clots to stop bleeding. Thrombocytopenia (or low platelet counts) is one of the most common hematological disorders in neonates, affecting 20-35% of infants admitted to the Neonatal Intensive Care Units (NICU).[1,2] This incidence is inversely proportional to the gestational ages of the infants, and reaches 70% among the smallest patients (<1000 grams).[3] In 25% of affected neonates, the platelet count drops below 50 x 10^9/L (severe thrombocytopenia), and 9% of those infants experience clinically significant bleeding (approximately 60% intracranial).[1,4] Thrombocytopenia is a known risk factor for clinically significant bleeding in neonates that often leads to life-long consequences (intracranial bleeding), however, there is a poor correlation between degree of thrombocytopenia and bleeding risk.[1,4,5] Emerging evidence supports that factors (such as gestational age, postnatal age, certain diagnoses, hematocrit, etc.) other than the platelet count significantly influence primary hemostasis as well as bleeding risk, and that better tests are needed to identify thrombocytopenic neonates who are at significant risk for bleeding. Although platelet transfusions are frequently given to neonates with platelet counts below a certain arbitrary trigger, it is unknown what truly constitutes a "dangerous" platelet count in this patient population and which neonate would truly benefit from a platelet transfusion. In addition to it and based on an association between the number of platelet transfusions and mortality and morbidity of NICU patients, several investigators have proposed that platelet transfusions could have deleterious effects in neonates.[6-10] While severe thrombocytopenia resolves within 14 days in 80% of affected neonates, in approximately 10% it persists for >30 days, resulting in multiple platelet transfusions (>20).[11,12]

Neonatal thrombocytopenia continues to be a common and potentially dangerous condition calling for not only better markers for transfusion decisions, but also new therapeutic options other than platelet transfusions.

2 BACKGROUND

2.1 NEONATES

A normal pregnancy lasts about 40 weeks or 280 days.[13] Full term is defined as being born between 37 and 42 gestational weeks, and preterm birth is below 37 gestational weeks. Preterm birth is further categorized into three subcategories:

1. moderate to late preterm (between 32 and 37 weeks),
2. very preterm (28 – 32 weeks), and
3. extremely preterm (less than 28 weeks) (**Figure 1.**).

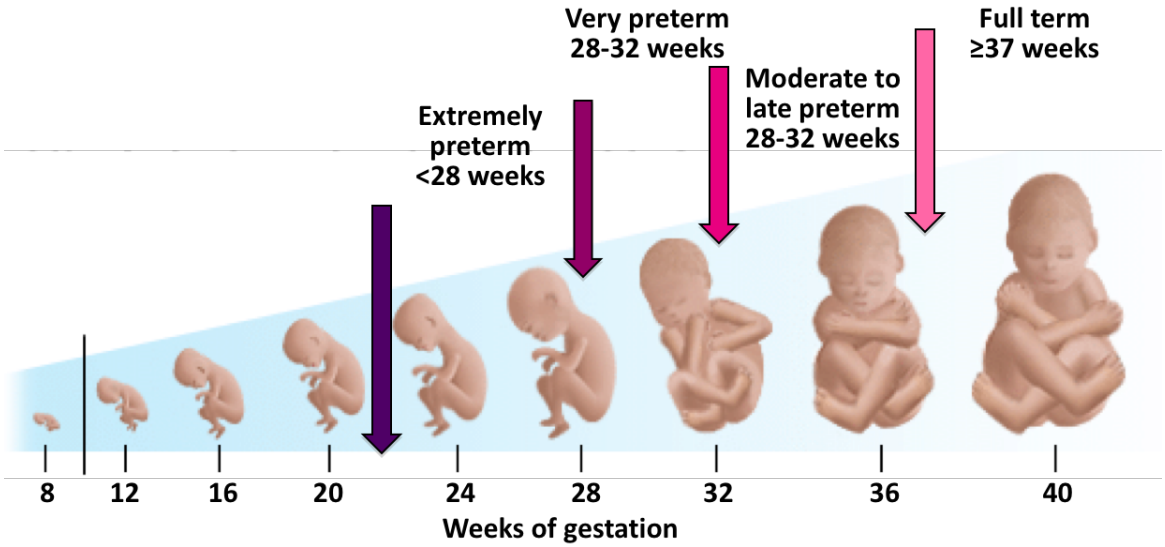

Figure 1. Fetal growth, definition of preterm and term births, and subcategories of preterm birth.

It is estimated by the World Health Organization that approximately 130 million babies are born each year and more than 10% (approximately 15 million babies) are born preterm. In Sweden, about 115 000 – 120 000 infants are born every year and around 6% of them are preterm (~7000). Complications associated with prematurity are the leading causes of death under 5 years of age: approximately 1 million children die yearly due to preterm birth. It is estimated that about 75% of these cases could have been prevented.[14]

While most of the newborns do not require special care, about 9% of all neonates in Sweden requires neonatal care and admission to the NICU, similarly to in the U.S.[15] Neonatal period indicates the time between birth and one month of age.

2.2 DEFINITION AND EPIDEMIOLOGY OF NEONATAL THROMBOCYTOPENIA

Thrombocytopenia is the second most common hematological disorder in neonates, affecting 20-35% of patients admitted to neonatal intensive care units (NICUs).[1,2] Neonatal thrombocytopenia is traditionally defined as a platelet count <150 x 10^9/L and is further classified as mild (100-149 x 10^9/L), moderate (50-99 x 10^9/L), or severe (<50 x 10^9/L). However, consistent with the data by Wiedmeier et al., platelet counts of 100–149 × 10^9/L are somewhat more common among neonates than adults. Large studies in unselected populations reported an overall incidence of neonatal thrombocytopenia of 0.7 to 0.9%,[16,17] however, among neonates admitted to the NICUs the incidence of thrombocytopenia is much higher and varies between 18 and 35%.[2] The incidence of thrombocytopenia is inversely proportional to the gestational age (weeks of gestation at birth) thus is highest among the most preterm infants reaching 73% in infants with a birth weight <1000 g.[3] The most recent and largest study demonstrated that platelet counts at birth increase with advancing gestational age. Importantly, while the mean platelet count was ≥ 200 x 10^9/L even in the most preterm infants, the 5th percentile was 104 x 10^9/L for those ≤ 32 weeks gestation, and 123 x 10^9/L for late-preterm and term neonates (**Figure 2.**).[18] These findings suggested that different definitions of thrombocytopenia should be applied to preterm infants. Accordingly, platelet counts between 100 and 150 x 10^9/L appear to be more frequent among otherwise healthy extremely preterm infants, than among full term neonates or older children/adults.

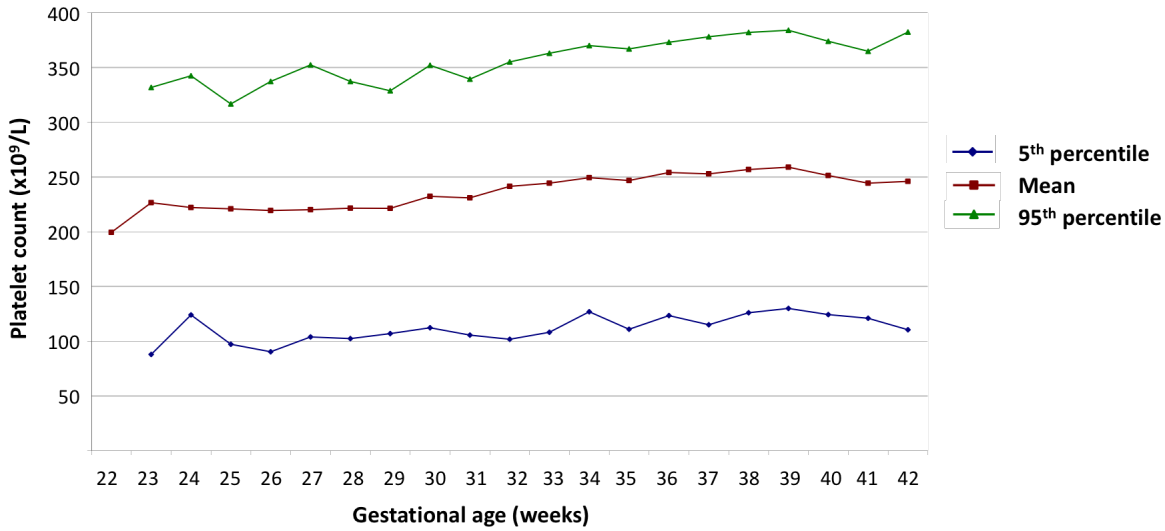

Figure 2. First platelet counts of infants at different gestational ages. All platelet counts were obtained in the first three days after birth. Mean values are marked with the red line, and the 5th and 95th percentiles are shown by the blue and green lines, respectively. (Adapted from: Christensen R, Henry E, Jopling J, and Wiedmeier S. The CBC: Reference ranges for neonates. Seminars in Perinatology 2009;33:6.)

2.3 ETIOLOGY OF NEONATAL THROMBOCYTOPENIA

The majority of thrombocytopenias in this patient group is acquired and due to non-immune mechanisms. The larger part of these cases resolves with time or with the treatment of the underlying diseases. Traditionally, thrombocytopenia in this patient population is classified based on the time of presentation and if the patient is sick or not.

The most common etiology of early-onset thrombocytopenia (<72 hours after birth) is placental insufficiency and chronic intrauterine hypoxia resulting in intrauterine growth restriction.[19,20] These infants are usually "well-appearing" and have transient and mild thrombocytopenia. Of note, several congenital infections (bacterial: syphilis; viral: cytomegalovirus, rubella, parvo B19 virus, human herpes simplex virus, HIV; parasitic: toxoplasma) can present with intrauterine growth restriction and thrombocytopenia. However, severe early-onset thrombocytopenia in a well-appearing infant should raise suspicion for immune thrombocytopenia (either alloimmune or autoimmune). Early-onset thrombocytopenia in sick neonates should warrant the evaluation for infection (bacterial, viral, parasitic, or fungal) or for disseminated intravascular coagulation. The most common etiology of late-onset thrombocytopenia (≥72 hours after birth) is sepsis (bacterial or fungal), necrotizing enterocolitis (NEC), or viral infections (acquired cytomegalovirus, enterovirus, adenovirus). These infants usually appear ill and have other associated findings. Other causes of late-onset thrombocytopenia may be thromboses (most often renal vein thromboses, or catheter-associated), or drug-induced (certain antibiotics, heparin, indomethacin, or phenobarbital)[21,22]. Rare causes of neonatal thrombocytopenia include inborn errors of metabolism, Fanconi anemia, or Kasabach-Merritt syndrome.

2.4 FETAL AND NEONATAL PLATELET PRODUCTION

During the last decade, several studies have clearly demonstrated that there are substantial morphological and biological differences between fetal/neonatal and adult megakaryocytes (MKs) and platelets. These developmental stage-specific differences are important, because they allow the fetus to maintain stable platelet counts while the blood volume is rapidly expanding, in a time period featured by exceptionally rapid growth. However, platelet production is highly complex, and can be described in four main steps (**Figure 3.**):

1. the production of thrombopoietic factors (mainly thrombopoietin or TPO),
2. the proliferation of MK progenitors,
3. the differentiation and maturation of MKs through a unique process of endomitosis, and finally
4. the production and release of platelets into the circulating blood.

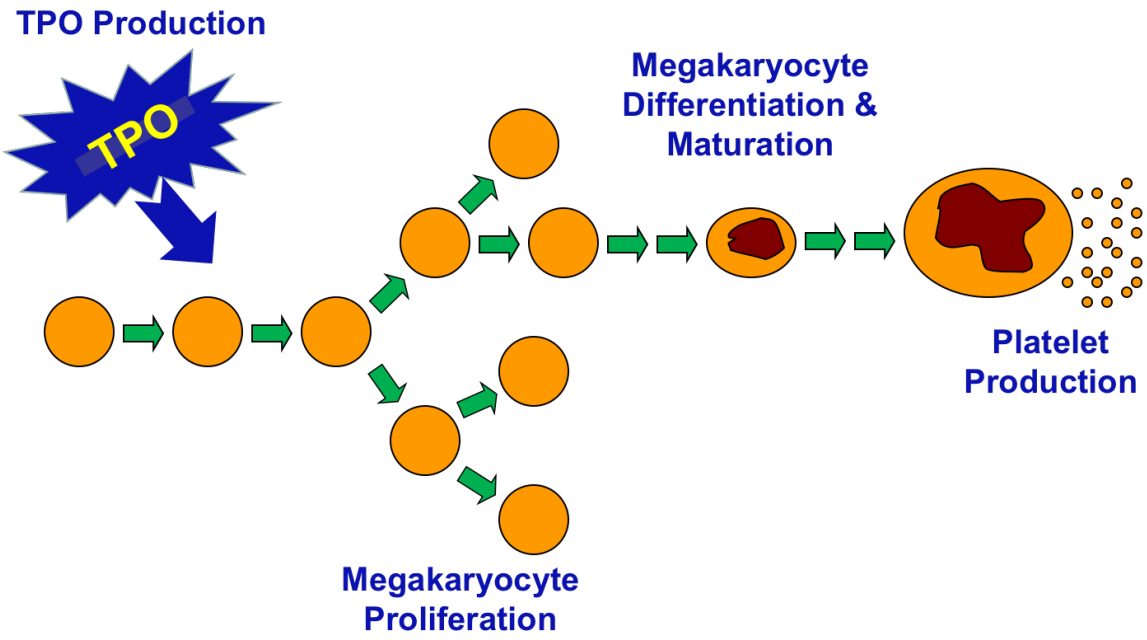

Figure 3. The four main steps of platelet production: TPO production, megakaryocyte progenitor proliferation, megakaryocyte differentiation and maturation, platelet release. (Adapted from Alarcón P, Werner E, Christensen RD. Neonatal Hematology: Pathogenesis, Diagnosis, and Management of Hematologic Problems, 2nd Edition, Chapter 11.)

Studies of MK progenitors derived from term and preterm umbilical cord blood, fetal blood (18-22 weeks gestation), or fetal bone marrow have shown that cultured fetal/neonatal MK progenitors proliferate at a significantly higher rate, compared to their adult counterparts. Additionally, fetal and neonatal MKs are substantially smaller and have lower ploidy levels than adult MKs (**Figure 4.**). Contrary to adult MKs, which mature as their ploidy level

increases, neonatal MKs are fully mature and capable of platelet production despite their small size and low ploidy. This dissociation between proliferation, polyploidization and cytoplasmic maturation is a distinctive characteristic of neonatal megakaryopoiesis. The net result of this process is the production of large numbers of low-ploidy but highly mature MKs, with which fetuses and neonates populate their rapidly expanding bone marrow space and blood volume, while maintaining normal platelet counts. As developmental processes are easily to disturb, however, sick neonates, and preterm infants, are at high risk of thrombocytopenia.

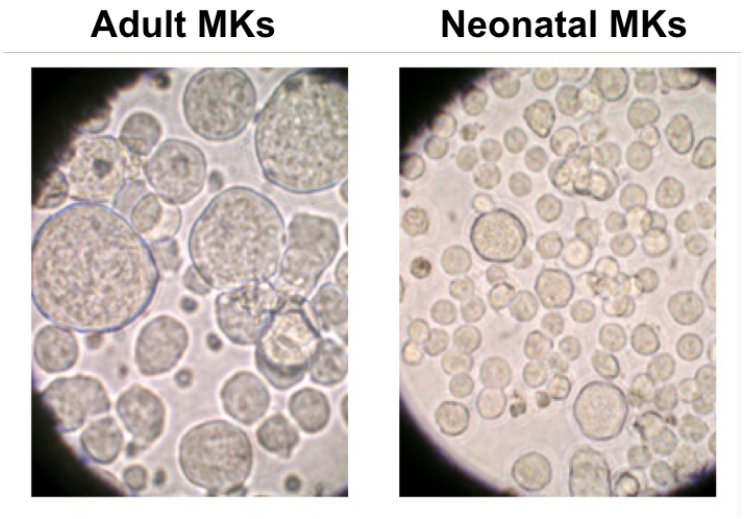

Figure 4. Photomicrograph of umbilical cord blood-derived (neonatal) and adult peripheral blood-derived (adult) megakaryocytes (MKs) at the end of a 14-day culture period (magnification of 600x). (Adapted from Liu ZJ, Sola-Visner M. Neonatal and adult megakaryopoiesis. Curr Opin Hematol. 2011 Sep;18(5):330-7.)

2.5 NEONATAL PLATELET FUNCTION AND PRIMARY HEMOSTASIS

Multiple studies evaluating platelet adhesion, aggregation and activation have shown that neonatal platelets are hyporesponsive *in vitro* to most agonists, compared with adult platelets,[23,24] and this hyporeactivity is more pronounced in preterm infants.[25,26] Platelet aggregation studies demonstrated that neonatal platelets (from full term umbilical cord blood) were less responsive than adult platelets to agonists such as adenosine diphosphate (ADP), epinephrine, collagen, thrombin, and thromboxane analogues.[27] Similar results were found in flow cytometric platelet activation studies, which showed decreased expression of surface activation markers in neonatal platelets stimulated with thrombin, ADP, and epinephrine.[25,27] Different mechanisms are responsible for the hyporeactivity of neonatal platelets to different agents: 1. the hyporesponsiveness to epinephrine is due to fewer α2-adrenergic receptors;[28] 2. the reduced response to collagen likely results from impaired calcium mobilization;[29] 3. the decreased response to thromboxane results from

differences in signaling downstream from the receptor in neonatal platelets;[23] and 4. the decreased responsiveness to thrombin is related to reduced expression of Protease Activated Receptor-1 (PAR-1) and PAR-4 in neonatal platelets.[30]

While the hypofunctional platelet phenotype *in vitro* would predict a bleeding predisposition, surprisingly, healthy full term neonates have normal to enhanced primary hemostasis evaluated by whole blood assays, compared to older children or adults. Bleeding times (BTs) in healthy term neonates are shorter than bleeding times in adults.[31] Similarly, studies using the Platelet Function Analyzer (PFA-100®, an *in vitro* test of primary hemostasis that measures the time it takes to occlude a small aperture, or Closure Time, **Figure 5.**) found that umbilical cord blood samples from term neonates demonstrated shorter closure times (CTs) than samples from older children or adults.[32,33] The results of these studies suggest that there is an enhanced platelet/vessel wall interaction in newborn infants born at term. The higher hematocrit, higher mean corpuscular volume, and higher concentration of von Willebrand factors (particularly its ultralong polymers) in neonatal than in adult blood[34] counterbalance the neonatal platelet hyporeactivity. In summary, the available evidence strongly suggests that the *in vitro* platelet hyporeactivity of healthy full term infants is an integral part of a carefully balanced and well-functioning neonatal hemostatic system, rather than a developmental weakness.

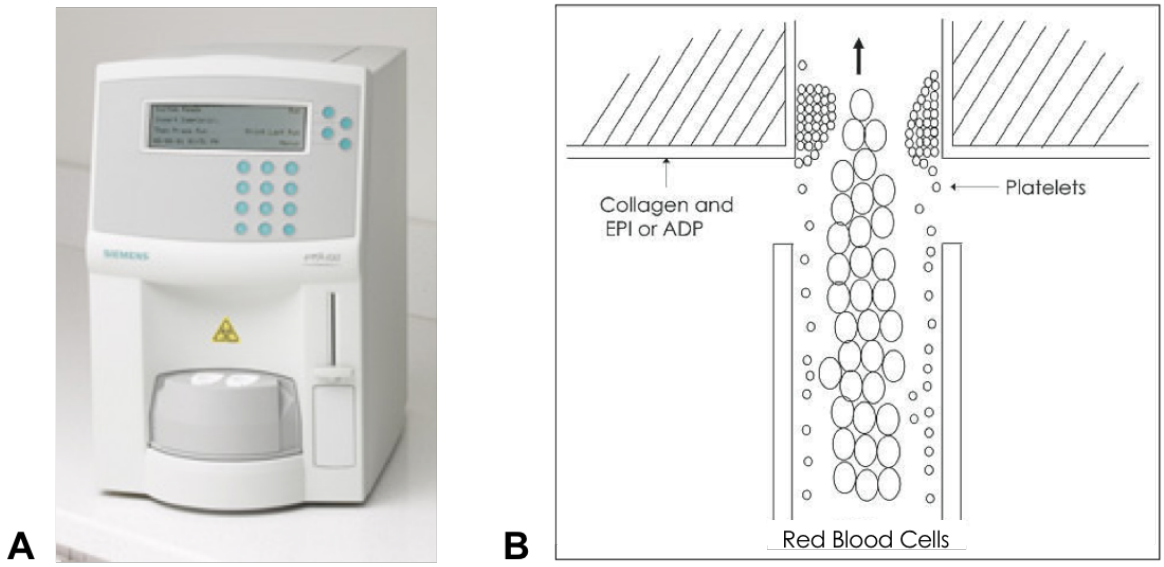

Figure 5.A) Platelet Function Analyzer-100 (PFA-100) is an easy-to-use bedside fast test of primary hemostasis that reports result in 5-8 minutes. **B)** Basic principle of the PFA-100: the PFA-100 measures the time it takes for blood to occlude an aperture (closure time, CT) following stimulation with collagen/epinephrine (CT-Epi) or collagen/adenosine (CT-ADP). (Adapted from Eugster M, Reinhart WH. The influence of the haematocrit on primary haemostasis in vitro. Thromb Haemost. 2005 dec;94(6):1213-8.)

The fine-tuned hemostatic balance might be less developed in preterm infants, whose platelets are already more hyporeactive than platelets from full term infants, leading to longer BTs.[35] Along with this, PFA-100 CTs from non-thrombocytopenic neonates were inversely correlated to gestational age in both umbilical cord blood and neonatal peripheral blood samples obtained on the first day of life.[36] Importantly, while these BTs and CTs were longer in preterm compared to term neonates, they were still near or within the normal range for adults, suggesting that healthy preterm neonates also have adequate primary hemostasis. Data regarding how disease processes (i.e. inflammation) perturb this delicate system, particularly in the preterm neonate, are lacking.

In vitro studies using flow cytometry or the cone and platelet analyzer showed that neonatal platelet function improves significantly and nearly normalizes by 10-14 days of postnatal age (studies included preterm infants).[25,26,37] Consistent with this, a study by Del Vecchio et al. found that, by 10 days of age, all infants had shorter BTs than at birth, and the initial gestational age-related differences had disappeared. Moreover, little or no further shortening occurred between days 10 and 30.[35] Notably, preterm neonates have the highest risk of bleeding during the first 10 days of life.[4]

2.6 THROMBOCYTOPENIA AND BLEEDING

25% of thrombocytopenic neonates have one or more platelet counts <50 x 10^9/L (severe thrombocytopenia), and 9% of these infants experience significant bleeding. Moreover, 87% of all clinically significant bleeding occurs in neonates <28 weeks gestation and <14 days of age.[1,4] Uniquely in this patient population, the risk of intracranial or intraventricular hemorrhage (IVH) is especially high; approximately 30% of neonates with birth weight <1500 grams suffer IVH, usually during the first week of life.[38] It is also known that thrombocytopenia is associated with an increased risk of IVH in the first week of life without correlation between the degree of thrombocytopenia and IVH risk.[5] The thrombocytopenia in these patients is most often transient and/or mild, requiring no intervention. However, in approximately 25% of the cases,[7,39] one or more platelet transfusions are ordered in an attempt to decrease the risk of hemorrhage, as a consequence of the high incidence of both thrombocytopenia and bleeding in the most preterm infants. Also, platelet transfusions are currently the only therapeutic option for neonates with non-immune thrombocytopenia. Despite this high prevalence, there have been insufficient evidence to guide platelet transfusions in newborn infants, with the platelet count being the only marker for the assessment of primary hemostasis.

2.7 PLATELET TRANSFUSIONS

Evidence, Benefits and Clinical Practice

As a result of the knowledge about the hyporesponsiveness of newborn platelets,[23,29,40] it has been widely accepted that newborn infants should be transfused at higher platelet counts than older children or adults. Until most recently, the strongest evidence came from only one randomized trial which was limited to very low birth weight (VLBW) infants in the first week of life, and excluded patients with severe thrombocytopenia (platelet counts <50 x 10^9/L).[41] This trial found no significant differences in the incidence or severity of IVH between VLBW neonates transfused for any platelet count less than 150 x 10^9/L and those transfused only for counts below 60 x 10^9/L. Based on these findings, the investigators concluded that transfusing VLBW infants with platelet counts >60 x 10^9/L does not reduce the risk of IVH. The relationship between degree of thrombocytopenia and bleeding risk has been assessed in a number of neonatal studies.[1,4] In the PlaNeT-1 study, it was found that the nadir platelet counts were similar in infants who experienced clinically significant bleeding compared to those with no or only minor bleeding.[4] A secondary analysis looking at all bleeding events (minor as well as clinically significant) found that a lower nadir platelet count was associated with only a slightly increased number of bleeding events. In this study, the strongest predictors of bleeding were gestational age <28 weeks, postnatal age <10 days, and the diagnosis of necrotizing enterocolitis (NEC).[42]

Due to lack of clinical trials, there have been substantial clinical practice variations worldwide in the use of platelet transfusions to treat thrombocytopenic neonates.[7,11,43,44] As a consequence, platelet transfusions in the NICU have been administered based on physicians' preferences, or in accordance with local guidelines arose from expert recommendations founded on limited scientific evidence. Survey data has suggested that neonatologists in the U.S. traditionally have transfused at much higher platelet counts than neonatologists in European countries, and this tendency has been estimated to result in the administration of 1.8 times more platelet transfusions in U.S. compared to European NICUs.[45]

Most recently (late 2018), PlaNeT-2, the biggest multicenter trial on prophylactic platelet transfusion thresholds in preterm infants reported its results.[10] A total of 660 patients with gestational age <34 weeks were randomly assigned either to the low-threshold (platelet counts <25 x 10^9/L) or to the high-threshold group (platelet counts <50 x 10^9/L). The median gestational age was 26.6 weeks and the median birth weight was 740 grams. The results showed a significantly higher rate of the primary composite outcome of death or major bleeding in the high-threshold group within 28 days after randomization than in the low-threshold group (26% vs. 19% respectively, OR=1.57 (1.06-2.32), p=0.02), similarly in

the subgroup analysis of infants <28 weeks of gestation. Of note, the median postnatal age at enrollment was 1 week and only 37% of the patients were enrolled ≤5 days of age and 59% ≤10 days of age. Additionally, 39% of the patients had received at least one platelet transfusion before randomization. In the secondary analyses, no significant differences in the rates of minor or worse bleeding were found, which is consistent with previous findings from observational studies showing a poor correlation between the degree of thrombocytopenia and bleeding.[4,42] Interestingly, the post hoc analyses showed a higher rate of bronchopulmonary dysplasia at 36 weeks of post-menstrual age in the high-threshold group than in the low-threshold group, as for the composite outcome of death or bronchopulmonary dysplasia.[10]

Side Effects and Potential Harms

Despite extensive donor screening, contamination of platelet suspensions with bacterial or viral pathogens (known and unknown) can still occur.[46] Bacterial infections, in particular, are more frequently associated with platelet transfusions than with any other blood product, due to the need to store this blood product at room temperature.[47] Furthermore, transfusion-transmitted infections with the potential for serious long-term consequences (i.e. Chagas disease)[48-50] are more relevant in neonates, whose expected lifespan is >70 years. Other transfusion complications, such as transfusion-associated lung injury (TRALI), are likely to be unrecognized and are probably underreported in neonates, who frequently have respiratory decompensation for other reasons. In the neonatal population, no study to date has demonstrated a beneficial effect of platelet transfusions. On the contrary, several publications have shown a strong association between platelet transfusions and high morbidity and mortality among NICU patients.[6-9] It is unclear from these studies whether this association simply reflects the fact that sicker patients receive more platelets, or whether platelet transfusions themselves adversely affect outcome, as has been suggested.[6] Most recently, PlaNeT-2 found an association between platelet transfusions and increased mortality and morbidity among preterm neonates: there was a significantly higher rate of death or major bleeding, and a higher rate of bronchopulmonary dysplasia when using more liberal strategy for platelet transfusions.[10] These reports highlight the need to make platelet transfusion decisions in neonates thoughtfully, carefully balancing the risks and benefits in each individual patient.

2.8 ASSESSMENT OF BLEEDING RISK

It has become increasingly clear that the bleeding risk is not directly correlated to the severity of thrombocytopenia, yet it is unclear to what degree other factors (such as the platelet size, gestational age, diagnosis, vascular immaturity or severity of illness) may contribute to the bleeding risk in neonates. Currently, there is a lack of good markers to assess bleeding risk in neonates with thrombocytopenia. A bleeding risk assessment marker could potentially help in estimating the risk/benefit ratio of transfusions, guiding platelet transfusion decisions in neonates with thrombocytopenia. Accurate new markers could potentially protect vulnerable infants who are currently being exposed to unnecessary transfusion-related risks. The PFA-100 is an *in vitro* test of primary hemostasis that provides a quantitative measurement of platelet adhesion, activation, and aggregation in whole blood.[51] As PFA-100 CTs represent global measurements of primary hemostasis, they are particularly attractive in neonates, since many factors contribute to their finely balanced hemostatic system.

2.9 THROMBOPOIETIC GROWTH FACTORS

Epidemiological data suggests that 10% of thrombocytopenic neonates have prolonged severe thrombocytopenia, lasting longer than 30 days and resulting in multiple platelet transfusions (>20).[11,12] Platelet transfusions are currently the only therapeutic option for neonates with long-standing thrombocytopenia, putting these neonates at risk for repeated transfusion. A strong association between the number of platelet transfusions and increased neonatal mortality and morbidity has been described.[9,43]

While severe thrombocytopenia resolves within 14 days in 80% of affected neonates, in approximately 10% it persists for >30 days,[11] and these infants are exposed to several platelet transfusions.[12] In 2008, two TPO mimetics, romiplostim (ROM) and eltrombopag (ELT), were approved in Europe and in the U.S. for the treatment of adults with chronic immune thrombocytopenic purpura and, more recently, ELT was approved for use in other varieties of chronic thrombocytopenia (chronic hepatitis C, aplastic anemia). In 2015, based on the positive results of PETIT2 - a randomized, multicenter, placebo controlled trial of eltrombopag in children ages 1 to 17 years[52] - ELT was approved in 2015 by the FDA for use in children with symptomatic chronic immune thrombocytopenia. Recent publications have reported successful use of ELT in adult and pediatric patients with various hematological disorders, including inherited thrombocytopenia associated with MYH9 mutations,[53,54] Wiskott-Aldrich syndrome,[55] and aplastic anemia.[56,57] Along these lines, it is plausible that ELT would also be considered as a therapeutic alternative in neonates, infants

and children in the first year of life with different varieties of thrombocytopenia. Both ROM and ELT begin to raise platelet counts 4-6 days after the initiation of the treatment and reach peak platelet counts at 10-14 days. Because of these pharmacodynamic characteristics, these drugs would be only appropriate for infants with prolonged severe thrombocytopenia. Based on the duration and severity of thrombocytopenia, a subset of thrombocytopenic neonates as well as infants with different varieties of thrombocytopenia could potentially benefit from TPO mimetics, but there is very limited data regarding the developmental stage-specific responses to these agents.

Our group previously described significant cellular and molecular differences between neonatal and adult megakaryocytes in their response to TPO,[58] suggesting that responses of thrombocytopenic neonates to TPO mimetics would be different than those of adults. As these agents could offer a new therapeutic approach to prolonged thrombocytopenia in neonates, establishing the safety and efficacy of TPO mimetics in this age-group is important.

3 AIMS

The overall aim of this doctoral project was to identify better markers for transfusion decisions in neonatal thrombocytopenia, and to contribute to establishing the safety and efficacy of new therapeutic options in prolonged neonatal thrombocytopenia other than platelet transfusions, consequently saving thrombocytopenic neonates from exposure to multiple transfusions.

Specific aims:

1. First, to determine the relationship between platelet counts and *in vitro* bleeding times (PFA-100 closure times - CTs) in thrombocytopenic neonates, and to determine what other factors significantly affect CTs. ***(Study 1, Paper I.)***

2. Second, to compare the strength of an association between platelet count and clinical bleeding with that of the association of *in vitro* bleeding time and clinical bleeding in thrombocytopenic preterm infants. ***(Study 2, Paper II. and III.)***

3. Third, to compare the responses of neonatal vs. adult human megakaryocyte (MK) progenitors to thrombopoietin (TPO) and two TPO mimetics, romiplostim (ROM) and eltrombopag (ELT), as these agents could offer new therapeutic approaches to prolonged thrombocytopenia in neonates. ***(Study 3, Paper IV.)***

4 STUDY DESIGN AND METHODS

4.1 STUDY DESIGN AND STUDY POPULATION

4.1.1 Study 1

This was a single institution prospective cross-sectional clinical study conducted in the NICU at Shands Children's Hospital at the University of Florida. Patients were eligible for the study if they had at least two platelet counts <150 x 10^9/L. Exclusion criteria were: major chromosomal anomaly (trisomy 13, 18, or 21); patient was thought to have congenital thrombocytopenia or platelet dysfunction, based on family history or clinical presentation; had received indomethacin within the previous 7 days; was on extracorporeal membrane oxygenation (ECMO); had a weight <750 grams; had received platelets or fresh frozen plasma between the two platelet measurements; or the attending neonatologist thought that the patient was unlikely to survive for >14 days.

4.1.2 Study 2

The Neonatal Hemorrhagic Risk Assessment in Thrombocytopenia Study (Neo-HAT Study) was a prospective longitudinal clinical study of thrombocytopenic preterm neonates in two academic centers (Astrid Lindgren Children's Hospital at Karolinska University Hospital, Stockholm, Sweden, and Levine Children's Hospital, Carolinas Healthcare System, Charlotte, NC, USA) between May 2015 and September 2017. Patients admitted to the NICU were eligible if they met the following criteria: had a gestational age <32 weeks or birth weight <1500 grams; and had a platelet count <100 x 10^9/L. Patients were excluded if they: were not expected to survive by the attending neonatologist; were thought to have a congenital thrombocytopenia or platelet dysfunction; or had a major chromosomal anomaly (trisomy 13, 18, or 21).

4.1.3 Study 3

This was a pre-clinical study using cell cultures. Hematopoietic progenitor (CD34+) cells isolated from umbilical cord blood (CB) from healthy full term neonates (38-42 weeks gestation) and adult mobilized adult peripheral blood (PB) were cultured in the initial experiments. The intracellular iron chelating effects of ELT were assessed in K562 cells (undifferentiated progenitor cells) and CB-megakaryocytes (CB-MK).

4.2 STUDY DATA AND PROCEDURES

4.2.1 Study 1 and Study 2

In *Study 1*, data was obtained from the medical records regarding the infants' gestational age, postnatal age, post-menstrual age at the time of study, and diagnosis. Blood samples (drawn according to protocol, see *Paper I.*) were tested on the PFA-100® with both collagen/ epinephrine (CT-Epi) and collagen/adenosine diphosphate (CT-ADP) cartridges. Hematocrit, platelet counts, and mean platelet volumes were also obtained on the same samples. Platelet mass was determined by multiplying the platelet count with the mean platelet volume.

In *Study 2*, the principal exposure variable was the CT-ADP, which was measured at three consecutive times using the PFA-100® (similarly to in *Study 1*), at the same times that platelet counts were checked (timing determined at the attending physician's discretion). Platelet counts were measured from the same blood draw as the CT-ADP, as part of the complete blood count run in the clinical laboratory. The primary outcome was bleeding, which was quantified in a scale of 0 (no bleeding) to 4 (severe bleeding), assessed at the same time as blood sampling using a previously validated Neonatal Bleeding Assessment Tool (NeoBAT) (**Table 1.**). The NeoBAT is a reliable and objective tool for the standardized assessment of bleeding in neonates, which was developed by an international team of experts based on the World Health Organization bleeding score for adults, and has been validated in NICU patients.[59] The bedside nurse recorded any evidence of bleeding for that day and any head ultrasound results from the last 24 hours were recorded in the NeoBAT form. Completed forms were collected by research nurses, who entered the data into the Neo-HAT database.

Grade 1 Minor Hemorrhage: any bleed from the • Skin, umbilical cord, skin around stoma, surgical scar, mucosa, • Any pink frothy or old bleed from the ET tube, • H1 hemorrhage on cranial US (Germinal Layer Hemorrhage, GLH).
Grade 2 Moderate Hemorrhage: any frank bleed from • the stoma, • macroscopic hematuria, • IVH (H2 or H3) without ventricular dilation (V0), • acute fresh bleed through ETT without ventilatory changes.
Grade 3 Major Hemorrhage: any • frank rectal bleed, • acute fresh bleed through ETT with ventilatory change, • major IVH: H2 or H3 with ventricular dilation (V1); H1, H2, H3 with parenchymal involvement (P3); any evolution of intracranial hemorrhage to H2V1, H3V1, or H1, H2, H3 with parenchymal involvement (P3).
Grade 4 Severe Hemorrhage: • Shock defined as life-threatening major bleed associated with hypotension, hypovolemia or any other hemodynamic instability and/or bleeding requiring volume boluses, red blood cell transfusion in the same 24 hours, fatal major bleeding.
H1=germinal layer hemorrhage; H2=intraventricular hemorrhage filling up less than 50% of the ventricle; H3= intraventricular hemorrhage filling up more than 50% of the ventricle; V0=no ventricle dilation; V1=ventricle dilation; P1=parenchymal echodensity; P2=porencephalic cyst; P3= periventricular leukomalacia

Table 1. NeoBAT scoring system: definition of bleeding grades.

4.2.2 Study 3

Hematopoietic progenitor or CD34+ cells from either source (CB "neonatal" or PB "adult") were plated and were cultured in serum-free medium (StemSpan, Stemcell Technologies) with increasing concentrations of rTPO, ROM and ELT, as previously described.[58] Cell cultured with 50 ng/mL TPO (TPO50) served as internal controls. $CD34^+$ cells from CB source were also cultured with ELT or the iron chelators deferoxamine (DFO) and deferiprone (DFP).

Megakaryopoiesis was analyzed using hemocytometer under the microscope and by flow cytometry on days 7 and 14 (proliferation, differentiation and maturation).[60] (**Figure 6.**, details see in *Paper IV*.)

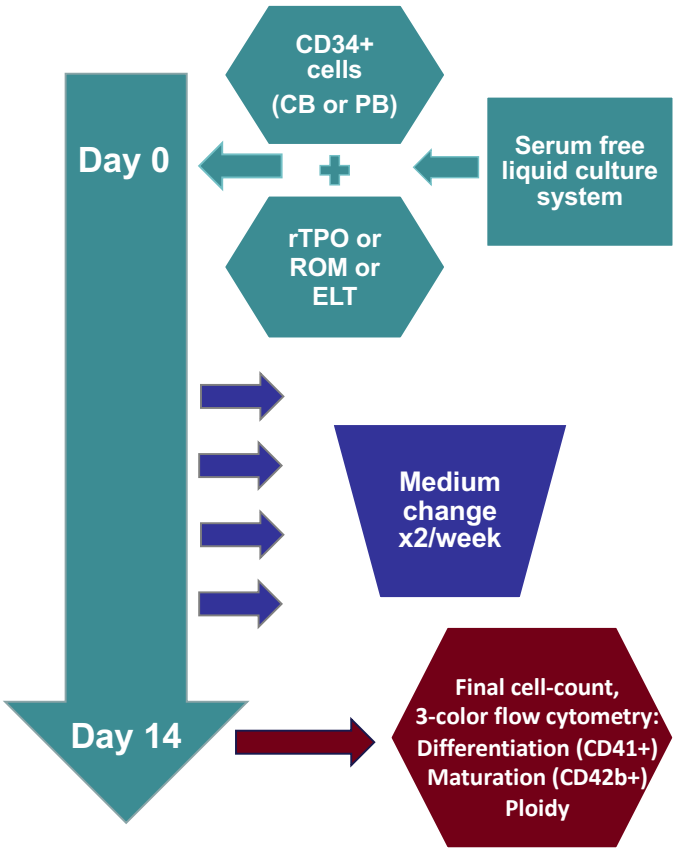

Figure 6. Initial experiments: full term umbilical cord blood (CB) or mobilized adult peripheral blood (PB) CD34+ cells were cultured in StemSpan medium with escalating concentrations of rTPO, ROM or ELT as solo growth factors.

The intracellular iron chelating effects of ELT were assessed in K562 cells and CB-MKs, using the calcein assay technique.[61] To evaluate the interaction between MK progenitors' iron status and ELT response, we changed our culture system to a self-made serum-free

culture system (IMDM-based medium)[58] with same transferrin concentration in all cultures (400 µg/mL), but with different holotransferrin (HOLO, iron-saturated)/apotransferrin (APO, iron-free) ratios (0% HOLO, 10% HOLO, 50% HOLO, and 100% HOLO).

On culture day 7, cells were split and cultured for an additional 72 hours with low TPO concentrations (3 ng/mL for CB-MKs, 10 ng/mL for PB-MKs) and with ELT or DFO. Megakaryopoiesis (proliferation, differentiation, maturation), Transferrin Receptor-1 (TFR-1) expression and apoptosis were assessed at the end of this 72-hour culture period. (**Figure 7.**, for further details see *Paper IV*.)

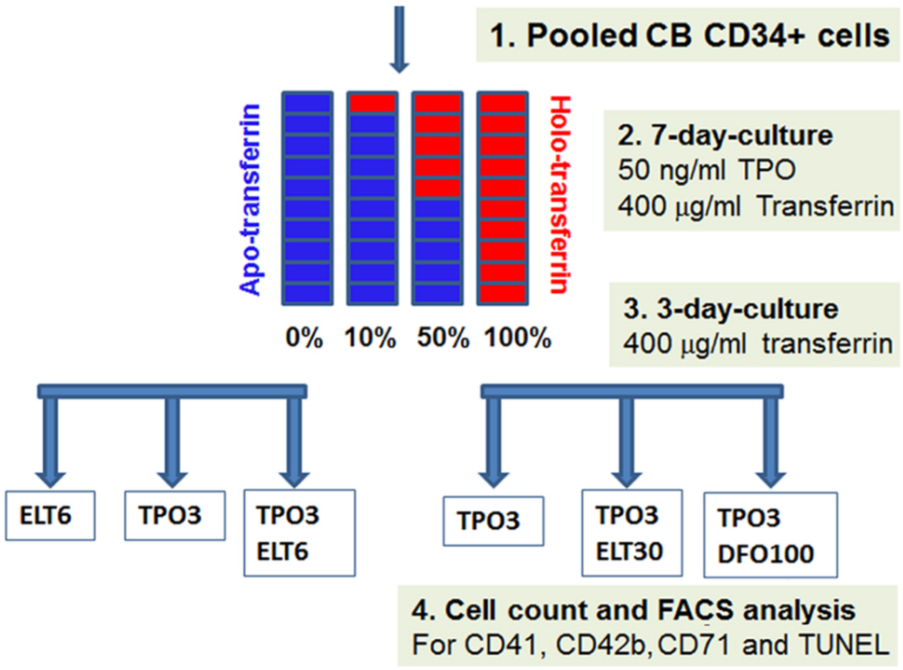

Figure 7. Iron preloading experiments: initially, full term umbilical cord blood (CB) CD34+ cells were cultured for 7 days in a serum-free culture system with the same transferrin concentration, but different ratios of apo- and holotransferrin to create iron-depleted, iron-deficient, and iron-replated CB-MK progenitors. After that, these MK-progenitors were exposed to TPO, ELT or deferoxamine (DFO). FACS analysis: flow cytometry analysis.

4.3 STATISTICAL METHODS

For *Study 2* the sample size was computed using http://biomath.info/power/. Assuming (from recent publications[4,42] and preliminary data[62]) that 9% of neonates with gestational age <32 weeks and platelet count <100 x 10^9/L will have major bleeding, a sample size of 72 would be able to detect a significant difference in CT-ADPs between patients with "moderate-to-severe" (2-4) and "none-to-minor" (0-1) bleeding at the 0.05 significance level and with a statistical power of 90%.

For *Study 1* and *Study 2,* the one-sample t-test to test for non-zero mean change and the two-sample t-test or Wilcoxon rank-sum test were used to compare means, as appropriate to the skewness of the distribution. For *Study 1*, correlations were examined between the CT-Epi and CT-ADP and the variables of interest: platelet count, platelet mass, SNAP II score, gestational age, and postnatal age. Both parametric and nonparametric estimates of correlation were assessed; where the distribution of the outcome measure was not normal, non-parametric results were used. Linear regression was used to determine the line of best fit. Regression was also used to examine the joint impact in the case of multiple significant correlations. For *Study 2,* repeated-measures regression analysis was used to quantify the association of bleeding score (dependent variable) with the simultaneous CT-ADP (independent variable) on 3 consecutive days in each patient, assuming unstructured within-patient covariance. The influence of demographic, hematologic, and clinical variables relative to CT-ADP was assessed by adding them to the regression model as covariates. In one instance of significant covariate interaction, we obtained stratum-specific estimates for the regression coefficient of CT-ADP. We used an analogous model to assess the relation between 1-day changes in bleeding score and 1-day changes in CT-ADP.

We took two-sided $p < 0.05$ as the critical value for statistical significance and used SAS software (version 9.3 for *Study 1* and version 9.4 for *Study 2*, Cary, NC) for all computations.

For *Study 3*, data were presented as mean ± SEM from at least 3 experiments. We used SAS software (version 9.4, Cary, NC) for all computations. To compare MK differentiation among culture conditions, and to compare the responses to changing iron concentrations under different culture conditions, we used mixed-model analysis of variance, with a random effect to account for within-donor correlation and Tukey-Kramer adjusted p-values to compensate for the multiple post-hoc tests. To compare cell expansion over 5 time points, we analyzed log-transformed values using repeated-measures analysis of variance to account for serial correlation with donor. (For details, see *Study 3/Paper IV*.)

4.4 INFORMED CONSENT AND ETHICS

Study 1 was approved by the Institutional Review Board (IRB) at the University of Florida, Gainesville, FL, USA and signed informed consent was obtained from the parents of every subject enrolled. *Study 2* received ethical permit from the Swedish Central Ethical Review Board and from the Carolinas Healthcare System Institutional Review Board, and signed informed consent was obtained from parents. All data were anonymized before data analyses and there was no risk of individuals being identified or coming to harm. In *Study 3*, human umbilical cord blood was collected from healthy full term neonates at Brigham and Women's Hospital Labor and Delivery with IRB approval (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA).

5 RESULTS

5.1 STUDY 1 AND STUDY 2

5.1.1 Patient Characteristics and Primary Outcome Measures

Study 1 included 48 neonates with thrombocytopenia: 8 term and 40 preterm (gestational age <37 weeks) infants. Mean gestational age was 30.9 ± 5.3 weeks (mean±SD), mean birth weight was 1744 ± 931 grams, and median postnatal age was 5 days (IQR 3-18). 68.7% of the neonates were male. Mean platelet count was 95 ± 28 x 10^9/L and mean hematocrit during the study was 42 ± 9%. Patient characteristics and hematological parameters, including hematocrit, platelet count, mean platelet volume, platelet mass (platelet count x mean platelet volume), CT-Epi and CT-ADP values, are displayed in **Table 2**.

Number of patients	48
Sex – male, n (%)	33 (68.7%)
Gestational age in weeks, mean ± SD	30.9 ± 5.3
Weight in grams, mean ± SD	1744 ± 931
Postnatal age in days, median (IQR)	5 (3 - 18)
Post-menstrual age in weeks, mean ± SD	32.5 ± 4.6
SNAP II score, median (IQR)	5 (4 - 10)
Hematocrit in %, mean ± SD	42 ± 9
Platelet count x10^9/L, mean ± SD	95 ± 28
Mean Platelet Volume in fl, mean ± SD	10.4 ± 2.7
Platelet mass in µl, mean ± SD	1025 ± 366
CT-ADP in sec, median (IQR)	79 (67.5 – 97.5)
CT-Epi in sec, median (IQR)	123 (97 – 229)

Table 2. Baseline patient characteristics in *Study 1*. Values for categorical variables are presented as n (%); values for continues variables are presented as mean ± SD if normally distributed, and as median (25-75 % IQR) if skewed.

In *Study 2*, 76 infants with a mean gestational age of 26.1 ± 2.4 weeks (mean±SD), a mean birth weight of 777 ± 310 grams, and a median postnatal age of 4 days (IQR 3-14) were enrolled. 57% of the neonates were male. Mean platelet count at inclusion was 67 ± 27 x 10^9/L and mean hematocrit during the study was 41 ± 5%. Patient characteristics and hematological parameters are displayed in **Table 3**.

Number of patients	76
Gestational age at birth in weeks, mean ± SD	26.1 ± 2.4
Birth weight in grams, mean ± SD	777 ± 310
Small for gestational age, n (%)	27 (36%)
Postnatal age at inclusion in days, median (IQR)	4 (3–14.5)
Post-menstrual age in weeks at inclusion, mean ± SD	27.9 ± 3.2
Sex, male, n (%)	43 (57%)
Platelet count x 10^9/L at inclusion, mean ± SD	67 ± 27
Hematocrit in % during the study period, mean ± SD	41 ± 5
CT-ADP in sec during the study period, mean ± SD	179 ± 88
SNAP II score during the study period, median (IQR)	5 (5–16)
NEC during study period, n (%)	25 (33%)
Sepsis during study period, n (%)	39 (51%)
Received penicillin during study period, n (%)	37 (49%)

Table 3. Baseline patient characteristics in *Study 2*. Values for categorical variables are presented as n (%); values for continues variables are presented as mean ± SD if normally distributed, and as median (25-75 % IQR) if skewed.

In *Study* 1, we examined the relationships between CT-Epi, CT-ADP, and the variables of interest: platelet count, platelet mass, SNAP II score, gestational age, and postnatal age. In the crude analysis, we found no significant association between CTs and gestational age, postnatal age, or SNAP II scores. Also, we did not find any association between CT-Epi and platelet count. However, there was a moderate negative correlation between CT-ADP and platelet count (r= -0.49, p= 0.0006). The scatter plot also suggested a threshold effect of platelet counts on the CT-ADP (**Figure 8.**). Almost all infants with platelet counts >90 x

10^9/L had CT-ADP values <100 seconds. Below a platelet count of 90 x 10^9/L, however, several infants had prolonged CT-ADPs (>100 seconds), although there was no linear correlation between platelet count and CT-ADP. Importantly, 7 out of the 9 patients who had platelet counts <90 x 10^9/L and CT-ADP values >100 seconds were ≤7 days old.

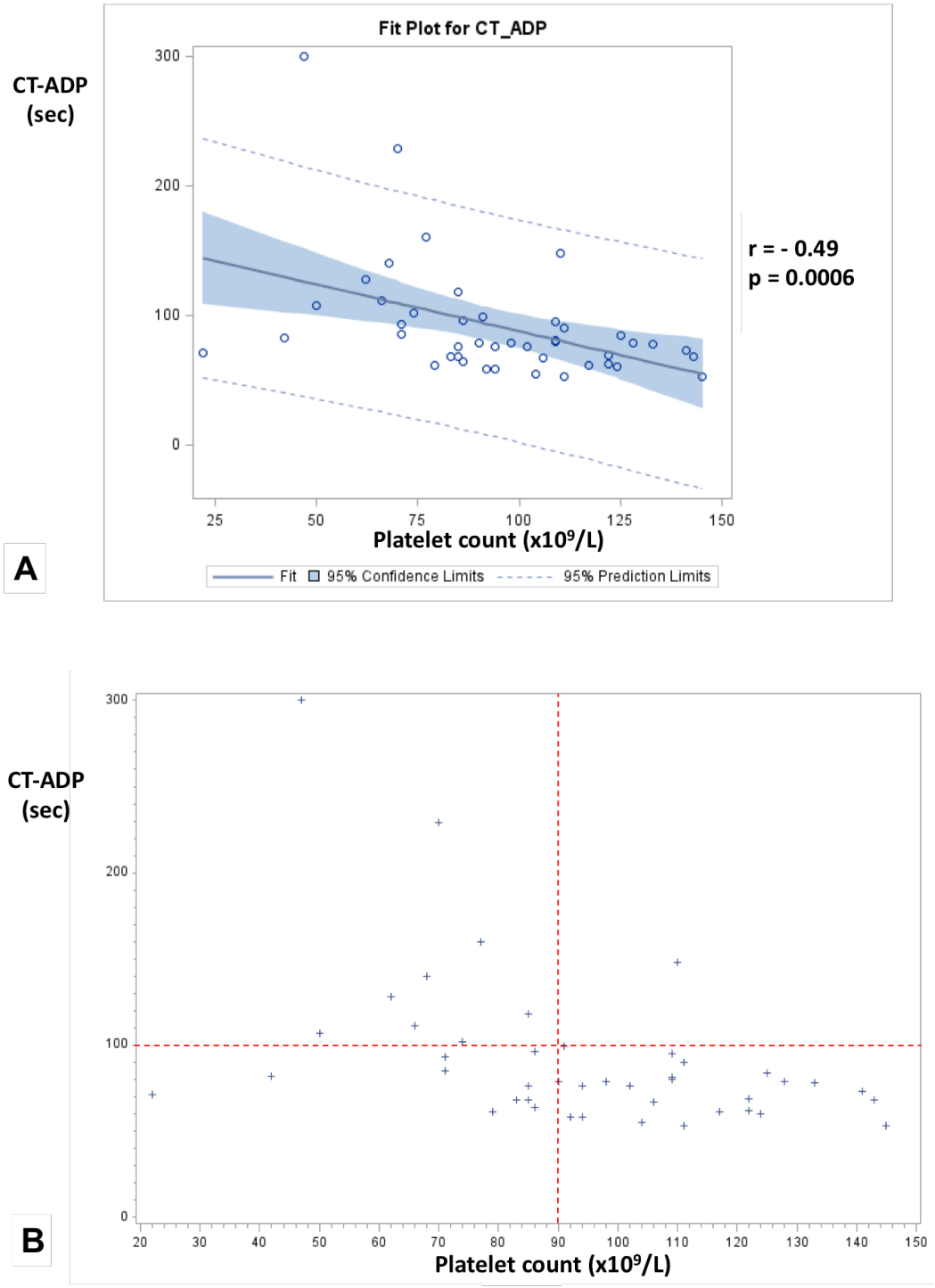

Figure 8. CT-ADP and platelet count association in all thrombocytopenic neonates: **A**) linear correlation between CT-ADP and platelet count; **B**) scatterplot with threshold effect of platelet count.

In *Study 2*, based on the findings of *Study* 1, we measured only CT-ADP. CT-ADPs and platelet counts were moderately inversely correlated (r= −0.37, p< 0.0001). Repeated-measures regression analysis showed the CT-ADP being associated with the NeoBAT score, with an effect size of 0.16 ± 0.05 score points per 60 seconds increase in CT-ADP (p= 0.003), but no significant association between platelet count and bleeding scores (effect size: -0.05 ± 0.04 per 10 x 10^9 /L platelet count increase, p= 0.20). When both CT-ADP and platelet count were included in the regression model, the platelet count had no association (p= 0.73), while CT-ADP remained a statistically significant predictor of NeoBAT score (p= 0.004). CT-ADP also remained robustly associated with bleeding score after controlling for clinically relevant covariates (gender, gestational and postnatal age, small for gestational age status, SNAP II score, NEC and sepsis diagnoses, hematocrit, transfusions and medications affecting platelet function such as penicillin, cephalosporin antibiotics, ibuprofen, indomethacin, paracetamol)[63-67]. The association between CT-ADP and bleeding score was limited to infants with gestational age <27 weeks (interaction p= 0.03), and lost statistical significance in infants ≥27 weeks.

5.1.2 Subgroup Analyses

In *Study* 1, as a next step, associations were examined in certain subgroups: preterm infants (gestational age <37 weeks), very preterm infants (gestational age <32 weeks), and infants <10 days old. A moderate negative correlation between CT-ADP and platelet count (n= 37, r= -0.49, p= 0.002) was found in the preterm group; and this negative correlation was even stronger in infants with gestational age <32 weeks (n= 26; r= -0.54, p= 0.0045), similarly to in infants with postnatal age <10 days (n= 29, r= -0.65, p= 0.0001). The strongest negative correlation was found in infants with gestational age <32 weeks and <10 days old (n= 12, r =-0.8, p= 0.0017), presumably representing a group with more homogeneous platelet function characteristics.

In *Study 2*, subsequent analyses focused on infants <27 weeks gestational age (n= 54). Bleeding episodes (grades 1-4) occurred more frequently in those infants than in infants ≥27 weeks (p= 0.01), and more were moderate-to-severe (grade 2-4): 34 of 99 (34%) vs. 1 of 45 (2%) (p< 0.0001). Infants <27 weeks also had longer median CT-ADPs than infants ≥27 weeks (196 vs. 136 seconds; p= 0.02), and a higher percentage of samples at the maximum 300 seconds (36% vs. 20%; p= 0.05). In repeated-measures regression analysis restricted to infants with gestational age <27 weeks at times when the platelet count was <100 x 10^9/L, bleeding scores did not correlate with platelet counts (p= 0.33) but were strongly associated with CT-ADPs (+0.28 point/60 seconds, p< 0.0001). Similar to findings for the entire cohort, the association of bleeding score with CT-ADP was not attenuated by adjustment for any of

the covariates examined. We did not find a statistically significant difference in overall NeoBAT scores between neonates ≤10 vs. >10 days old. However, among infants ≤10 days old, 8 of 91 (9%) of bleeding scores recorded were grades 3 and 4, compared to only 1 grade 3 bleeding among 53 NeoBAT scores in infants >10 days old (2%; p= 0.15).

5.1.3 Association Between Change of In Vitro Bleeding Time with Change of Bleeding Score and Effect of Transfusions

In *Study 2*, we examined whether changes in CT-ADP or platelet count between two consecutive days were accompanied by changes in NeoBAT score in infants with gestational age <27 weeks. Change in bleeding score showed a moderate positive correlation with the concurrent change in CT-ADP (r= 0.37, p= 0.006), but not with change in platelet count (r=0.03, p= 0.79). Notably, a decrease in CT-ADP in a given patient almost never coincided with an increase in the bleeding score (**Figure 9.**).

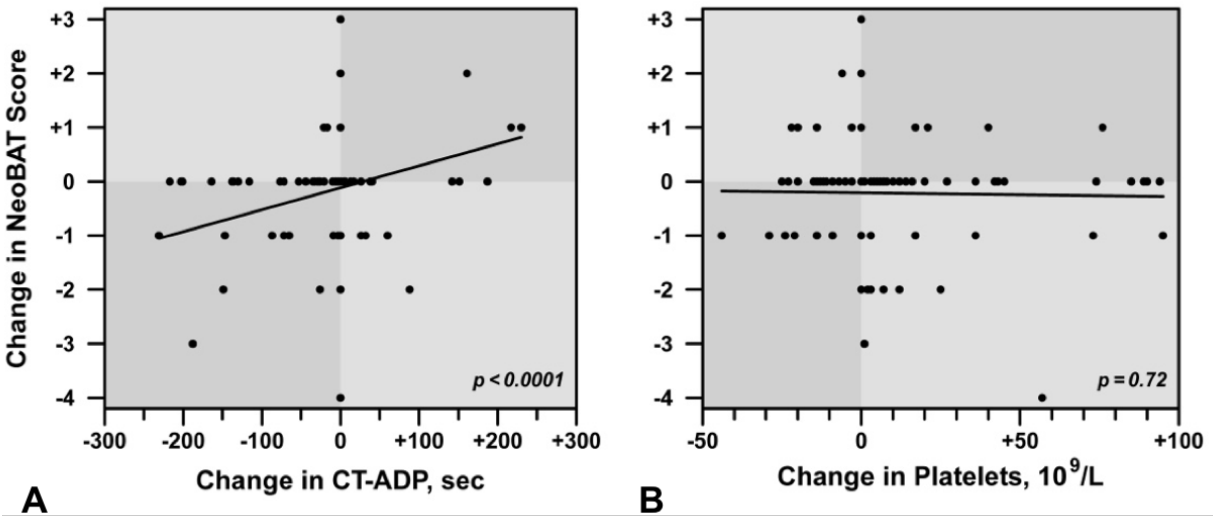

Figure 9. One-day change in NeoBAT score vs. change in CT-ADP (**A**) and vs. change in platelet count (**B**) in infants with gestational age <27 weeks. The fitted line was generated from repeated-measures regression analysis.

As the last step, the effects of platelet transfusion on changes in platelet count, CT-ADP, and bleeding score in the entire study cohort, including infants with gestational age ≥27 weeks were examined. Patients who received a platelet transfusion showed a significant one-day increase in platelet count (by a mean of 34 x 10^9/L) and a significant decrease in CT-ADP (by a mean of 39 seconds), but a small and marginally significant change in bleeding score. Compared to the changes observed in neonates who were not transfused, only the change in platelet count was significantly different between the two groups. Transfusions of FFP,

erythrocytes, or any blood product were not associated with a difference in changes in NeoBAT score or CT-ADP. The same analysis was performed in infants <27 weeks, with similar results. (For details, see *Paper III.*)

5.1.4 Bleeding Prediction

We examined the relationship between the CT-ADP and the following day's bleeding score using multinominal regression analysis and found the probability of moderate-to-severe (grade 2 or higher) bleeding on the following day rose with higher CT-ADP, from 10% at the minimum to 40% at the maximum (**Figure 10**). The probability of major-to-severe (grade 3-4) bleeding on the next day was notably low, rising from 2% at the minimum CT-ADP to 14% at the maximum (**Figure 10.**). The etiology of major-to-severe bleeding (grade 3-4) in this patient population was heterogeneous, but all of them revealed acute illnesses: 2 with NEC (one surgically and one medically treated), 3 with sepsis, 2 with pulmonary hemorrhage, and 2 with intraventricular hemorrhage (one isolated, the other with sepsis).

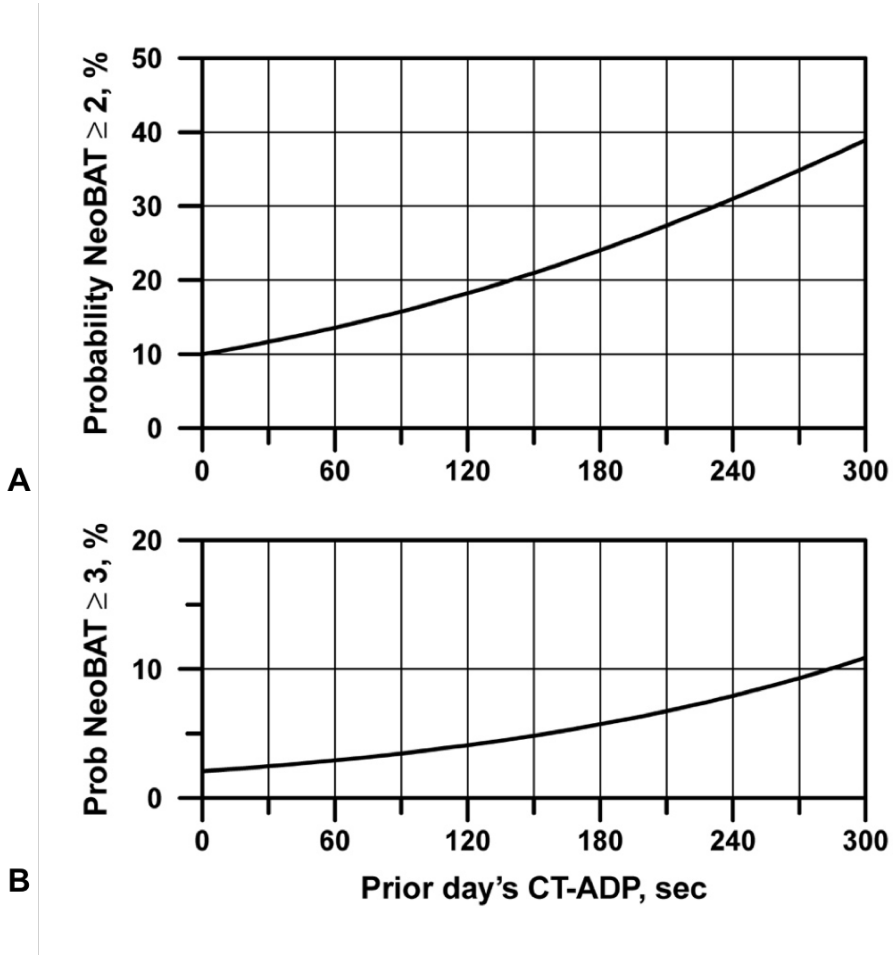

Figure 10. Multinominal regression model of NeoBAT vs. preceding day's CT-ADP: (**A**) the probability of moderate-to-severe bleeding (NeoBAT ≥ 2) vs. preceding day's CT-ADP, and (**B**) the probability of major-to-severe bleeding (NeoBAT ≥ 3) vs. preceding day's CT-ADP.

5.2 STUDY 3

5.2.1 Megakaryocyte Responses to Growth Factors: TPO, ROM and ELT

CB MK ("neonatal") progenitors proliferated at a higher rate than adult PB ("adult") progenitors and generated approximately 10 times more MKs (per $CD34^+$ cell plated) than adult PB in response to any of the thrombopoietic agents tested. For both cell sources, however, the maximal number of MKs generated in cultures with ELT was significantly lower than that achieved with TPO (p= 0.03 and 0.002 for CB and PB, respectively) or ROM **(Figure 11.A)**. At all TPO, ROM, or ELT concentrations, CB MKs exhibited significantly lower ploidy levels **(Figure 11.B)**, but higher percentages of CD42b+ MKs (a marker of mature MK) than PB cultures **(Figure 11.C)**. Notably, ploidy and CD42b surface expression levels in CB MKs were similar at all TPO and TPO mimetic concentrations tested.

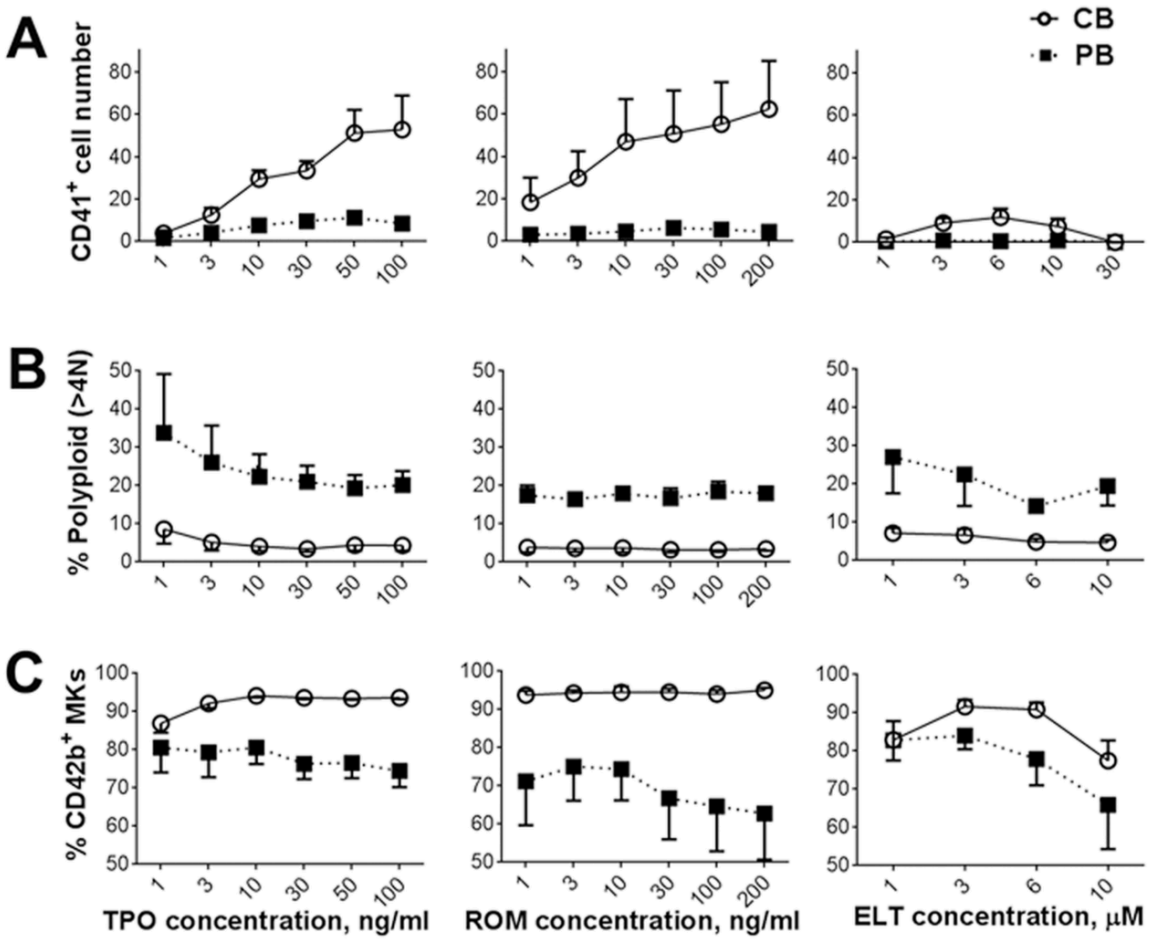

Figure 11. MK proliferation, polyploidization, and maturation in response to TPO, ROM, and ELT. **A)** Number of CD41+ cells (per CD34+ cell plated) generated from CB and PB in the presence of TPO (left), ROM (center), and ELT (right), measured after 14 days of culture. **B)** Percent of polyploid MKs (>4N), and **C)** percent of $CD41^+$ MKs that expressed CD42b generated in cultures with escalating concentrations of TPO, ROM, and ELT. Bars represent the mean ± SEM of 3-5 independent experiments.

Overall, ELT cultures generated less MKs than TPO or ROM. In this culture system, ELT concentrations >6 μM consistently yielded lower MK counts, consistent with a prior study showing that ELT concentrations of 6 μg/mL (13.6 μM) profoundly reduced the number of CB MKs generated *in vitro*.[68] The toxic effects of high ELT concentrations were not abolished by the addition of TPO at "high-physiological" concentrations found in hyporegenerative thrombocytopenias (3 ng/mL,TPO3) **(Figure 12.)**.

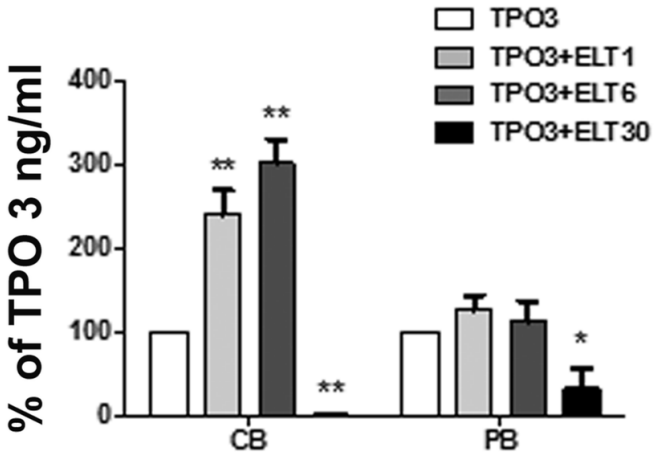

Figure 12. Combinational effects of ELT and low dose TPO. CD34+ cells derived from CB (**left**) and PB (**right**) were cultured in StemSpan medium supplemented with 3 ng/ml TPO alone or in combination with low (1 and 6 μM) or high (30 μM) concentrations of ELT for 14 days. Cultures with high ELT concentrations generated significantly less MKs than those with low or no ELT, particularly in CB-MKs. Data (n=3) is presented as percent change compared to TPO3 alone (which was set as 100%). *p<0.05; **p<0.01 vs. TPO3.

5.2.2 ELT and Megakaryocyte Intracellular Iron

ELT has structural homology with iron chelators, and has previously been shown to act as a potent iron chelator in leukemic cells,[69] cardiomyocyte cell lines[70] and neonatal hippocampal neurons[71]. These observations led us to investigate if the anti-proliferative effects of high ELT concentrations on MKs were associated with evidence of intracellular iron depletion. The free intracellular iron was quantified using calcein-AM, an intracellular iron-binding compound whose fluorescent signal is quenched when bound to Fe^{2+}. We confirmed that ELT depleted iron in a dose-dependent manner, indicated by progressive increase in calcein fluorescence activity. Moreover, ELT acted as a strong iron chelator (compared with a classical iron chelator as deferiprone or DFP). For details, see *Paper IV*.

To test whether the toxic effect of high ELT doses were due to iron depletion, CB ("neonatal") CD34+ cells were cultured with "high-physiological" concentrations of TPO (3 ng/mL,TPO3) alone or with the combination of TPO3 + ELT (6 and 30 µM), two iron chelators: DFO or DFP (6, 30 and 100 µM) for 14 days. TPO3 + ELT6 stimulated cell growth and achieved 2.4-fold higher cell number above TPO3, while ELT30 (**Figure 13.**) suppressed cell expansion from day 7, similarly to in response to DFO100 or DFP100, consistent with the stronger iron-chelating properties of ELT shown in the calcein studies.

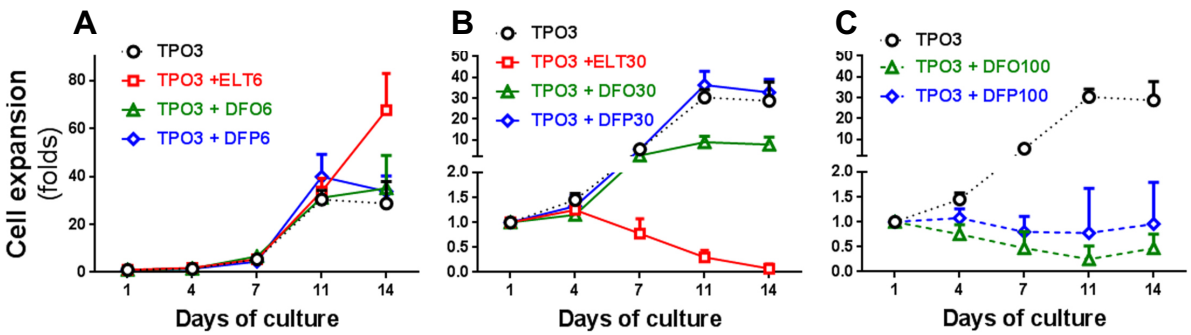

Figure 13. Dose-dependent iron-chelating effects of ELT on megakaryopoiesis. CB-CD34+ cells were cultured with TPO3 alone or TPO3 + the indicated concentrations (µM) of ELT or the iron chelators deferoxamine (DFO) or deferiprone (DFP) for 14 days. Cells were counted biweekly at the time of media changes, and data shown is the mean of 4 independent cultures. Compared to TPO3, ELT6 significantly stimulated cell growth (**A**) while ELT30 suppressed cell growth (**B**), similarly to DFO100 or DFP100 (**C**).

As for MK differentiation, we found that the percentage of mature MKs decreased substantially after 7 days in culture with ELT30, similarly to with DFO100 and DFP100. This finding appeared with an increase in the percentage of undifferentiated progenitors ($CD34^+/CD41^-$ cells), consistent with the observations from a recent study.[72]

5.2.3 Iron Status and ELT Effects on CB-MKs (Neonatal Megakaryocytes)

To determine whether the iron status of MK progenitors influences their response to ELT, we generated iron-depleted, iron-deficient, and iron-repleted MK progenitors (see in the Methods, **Figure 7.**).

In the first part of these experiments, after cells were cultured with different ratios of apo- and holotransferrin for 7 days, cells showed similar proliferation and differentiation regardless of iron content of the medium. However, nearly all $CD41^+$ cells on day 7 expressed surface Transferrin Receptor-1 (TfR-1), and the expression level inversely correlated to iron status. We then exposed these CB hematopoietic progenitor cells to an additional 72 hours in the

same culture medium, but supplemented with a "high-physiological" concentration of TPO (TPO3), low dose ELT (ELT6), or both (see in the Methods, **Figure 7.**). Cell expansion was correlated to the iron content in the cultures containing ELT6, however iron depletion did not affect cell expansion in response to low TPO concentrations (3 ng/ml), but to higher TPO concentrations (50 ng/ml).

Next, we carried out similar experiments to determine whether iron status influenced the proliferation of MKs cultured with high ELT concentrations (see in the Methods, **Figure 7.**). In iron-depleted cultures, high ELT concentrations (ELT30) and iron-chelator deferoxamine (DFO100) similarly impaired MK expansion and induced a 3- to 4-fold increase in MK apoptosis. However, the presence of holotransferrin (iron) at any concentrations in the cultures diminished these effects. In contrast to CB-MKs (neonatal megakaryocytes), we did not see the same pattern in adult PB-MKs (adult megakaryocytes). (See *Paper IV*.)

6 DISCUSSION

In our first study (*Study 1*), we evaluated *in vitro* primary hemostasis in thrombocytopenic neonates using the PFA-100. Our findings of a moderate negative correlation between *in vitro* bleeding time (CT-ADP) and platelet counts and a possible threshold effect of platelet counts on the CT-ADP were consistent with previous findings. A study by Andrew et al. found that the *in vivo* bleeding time was inversely correlated to the platelet count ($r= -0.56$, $p< 0.001$) and became prolonged when the platelet count fell to $<100 \times 10^9$/L.[1] Our results were similar to this: almost all infants with platelet counts $>90 \times 10^9$/L had CT-ADP values <100 seconds and when the platelet count fell below 90×10^9/L, some infants had prolonged CT-ADPs. These findings are also in line with that in the PLADO trial: the bleeding risk of pediatric patients with chemotherapy-induced thrombocytopenia substantially increased when the platelet count fell below 80×10^9/L, but without an obvious correlation between platelet count and bleeding risk.[73,74]

Our findings, that the strongest correlation between platelet count and CT-ADP was found in the group of infants <32 weeks gestational age and <10 days of life, indicate that gestational age- and postnatal age-related differences in platelet function may explain some of the bleeding variability found in neonates with thrombocytopenia. This rationale is supported by the findings of a recent prospective observational study of neonates with platelet counts $<60 \times 10^9$/L by Stanworth et al., that gestational age <34 weeks, postnatal age <14 days, and the presence of sepsis or necrotizing enterocolitis (NEC) were more important predictors of clinically significant bleeding than the platelet count alone.[4] Overall, these findings suggest that factors other than the platelet count substantially affect primary hemostasis as well as bleeding risk.

We did not find any correlation between platelet count and CT-Epi. This finding is in line with our previous study in non-thrombocytopenic infants, in which no correlation between gestational age and CT-Epi was found.[36] The most likely explanation for these observations is the sparse α2-adrenergic receptors on neonatal compared to adult platelets, which results in pronounced hyporesponsiveness to epinephrine.[75]

The clinical significance of the elevated *in vitro* bleeding time (CT-ADP) as well as the correlation between CT-ADPs and bleeding in thrombocytopenic neonates remained to be studied and initiated *Study 2*.

Thrombocytopenic preterm neonates have a high incidence of bleeding,[4,74,76] which has likely been a contributing factor to the liberal use of platelet transfusions in the NICU.[5,45]

However, several recent studies found a poor correlation between severity of thrombocytopenia and incidence of bleeding in various patient populations (including neonates), highlighting the limitations of the current platelet count based approach to transfusion decisions.[4,5,39,74,77] Our finding of the lack of association between the platelet counts and bleeding scores was consistent with those previous reports. The main finding of *Study 2* was that the CT-ADP (an objective test of primary hemostasis that reflects the contribution of multiple factors beyond the platelet count) was a significantly better marker of bleeding than the platelet count, particularly among neonates with the lowest gestational ages.

Neonates with gestational age <27 weeks, in whom the association between CT-ADP and bleeding scores was strongest, had a significantly higher frequency of moderate-to-severe (grade 2-4) bleeding than their more mature counterparts. Interestingly, we found that even moderate (grade 2) bleeding was infrequent among infants ≥27 weeks gestational age. This was consistent with the results of the PlaNET I study, which identified infants with gestational age <28 weeks as the neonatal population at highest risk of bleeding.[42] The reasons underlying the higher incidence of bleeding of extremely preterm infants are likely multifactorial, and involve *in vivo* factors that cannot be detected by a laboratory test. However, infants <27 weeks also had significantly longer CT-ADPs compared to infants ≥27 weeks and a higher percentage of samples measuring 300 seconds (the highest reported value), indicating a propensity to inadequate primary hemostasis that might contribute to their bleeding risk.

Platelet transfusions increased the next-day's platelet count, however, these transfusions had smaller and less significant effects on CT-ADP and bleeding scores. Also previous studies have found a lack of efficacy of platelet transfusions to decrease bleeding in neonates.[5,41] Such results may be interpreted as a proportionally smaller contribution of the platelet count to increasingly complex outcomes, which our study was not powered to address.

In the past 10 years, in adults, the use of TPO mimetics has gradually increased in different types of thrombocytopenia. Theoretically these agents could benefit thrombocytopenic neonates by potentially reducing platelet transfusions.

As shown in prior studies, CB (neonatal) megakaryocyte (MK) progenitors proliferated at a higher rate than adult PB MK progenitors and generated approximately 10 times more MKs than PB cultures in response to any of the thrombopoietic agents tested. At all TPO, and TPO mimetic (ROM or ELT) concentrations, CB MKs also exhibited significantly lower ploidy levels, but had higher percentages of mature (CD42b+) MKs than PB cultures. This was consistent with the previously described developmentally unique pattern of neonatal

megakaryopoiesis, characterized by rapid proliferation and full cytoplasmic maturation, without the need for polyploidization.[60,78] Ploidy and CD42b surface expression levels in neonatal CB MKs were similar at all TPO and TPO mimetic concentrations tested, reflecting the ability of neonatal MKs to undergo rapid maturation. PB MKs, in contrast, had a tendency toward lower ploidy and lower CD42b expression levels with rising concentrations of TPO mimetics, suggesting that at high TPO concentrations the rate of proliferation exceeded that of endomitosis and terminal maturation in adult MKs. Taken together, these studies suggested that the type and concentration of TPO mimetic primarily regulate the number of MKs generated in culture, but not their ultimate ploidy and maturational level, which are largely determined by the developmental origin of the progenitor (neonatal or adult).

Our finding that ELT concentrations >10 μM did not support the proliferation of neonatal CB hematopoietic progenitor cells was consistent with a previous report, [68] although the mechanisms mediating this toxicity remained unknown until now. In 2012, Roth et al. showed that ELT has structural homology with iron chelators and suppresses leukemic cell proliferation by decreasing intracellular iron. In that study, the antiproliferative effects of ELT were only observed in malignant cells and were thought to represent a selective anti-cancer effect.[69] Most recently, two other studies have shown the ELT act as a potent iron chelator even in cardiomyocyte cell lines[70] and in neonatal hippocampal neurons.[71] Our observations that the anti-proliferative effects of high ELT concentrations on CB MKs: 1. are associated with a reduction in intracellular iron (evidenced by increased calcein fluorescence and increased transferrin receptor, TfR-1 expression), 2. are attenuated if cells are preloaded with iron, and 3. are reproduced by iron chelators (deferiprone, deferoxamine) strongly suggest that the iron-chelating effects of ELT can also affect the proliferation of neonatal CB MK progenitors. Our data also support the conclusion that the iron-chelating effects of ELT are dose-dependent, and that the proliferative response of neonatal CB MKs to ELT is determined by the interplay of intracellular iron status and ELT dose.

Adult (PB) MK progenitors were not as sensitive to the antiproliferative and apoptotic effects of high ELT concentrations as neonatal CB MKs, even if iron-depleted. These findings suggest that these differences in response to ELT are likely related to higher iron requirements associated with the much higher proliferative rate of neonatal CB- compared to adult PB MK progenitors.

Taken together, our data suggest that decreased megakaryopoiesis would primarily be observed in the setting of highly proliferative MK progenitor cells (namely in infancy and early childhood) exposed to severe intracellular iron deficiency (potentially triggered by an

intracellular iron chelator in previously iron-deficient cells). This hypothesis is supported by several case series in the literature reporting thrombocytopenia in children with very severe iron deficiency, which resolved with iron administration.[79] The ELT concentrations we used in our culture systems were within the range achieved clinically.[56,80]

Limitations

We acknowledge that our studies have limitations. In *Study 1*, the sample size was small, as well as the number of patients with severe thrombocytopenia (platelet counts <50 x 10^9/L), nevertheless, this study served as important initial steps toward larger studies involving more patients and more blood samples. *Study 1* was not aimed to evaluate the clinical significance of *in vitro* bleeding time (PFA-100 CT-ADP). As a continuation, *Study 2*, however, evaluated the association between CT-ADP and clinical bleeding.

Study 2 also has some limitations. First, we had a small number of infants with severe thrombocytopenia (platelet counts <50 x 10^9/L), due to platelet transfusion practices in our NICUs, and a small number of patients with major-to-severe (grade 3-4) bleedings, as our study was powered for moderate-to-severe (grade 2-4) bleeding. We also lacked coagulation laboratory values in many patients. Finally, the CT-ADP does not incorporate important *in vivo* determinants of bleeding risk, such as vessel wall integrity, inflammation, and hemodynamic status. This likely sets a limit to the sensitivity and specificity that any *in vitro* test can achieve in the assessment of bleeding risk in this population.

We recognize the limitations of *Study 3*. Most importantly, it is an *in vitro* study, and therefore does not incorporate the complexities of the *in vivo* environment. Considering our findings of the toxic effect of high ELT concentrations on neonatal CB MKs, the potential effects of ELT on other neonatal organ systems with high iron requirements will also need to be carefully considered.

Furthermore, we recognize that all associations presented here may be subject to residual or unmeasured confounding variables due to the observational design of the studies, and that causal relationships have not been established.

7 CONCLUSIONS

- In *Study 1*, we measured *in vitro* bleeding time or PFA-100 closure times (CTs) in thrombocytopenic neonates of various gestational and postnatal ages and showed that CT-ADP was a better marker for evaluating neonatal platelet function than CT-Epi and that there was a moderate negative correlation between CT-ADP and platelet count. In addition, we found that there was a threshold effect of platelet counts on CT-ADP: all infants with platelet counts >90 x 10^9/L had normal CT-ADPs, and most infants with platelet counts <90 x 10^9/L had normal or minimally prolonged CT-ADPs, but a few exhibited significant prolongations.
- Also, the strongest correlation between platelet count and CT-ADP was found in the group of infants <32 weeks gestational age and <10 days of life, a subset of neonates with presumably more similar platelet function. These findings suggest that NICU patients are a heterogeneous population in terms of platelet function and primary hemostasis, which explains some of the variability in bleeding times and CTs found by us and others.
- *Study 2* showed that platelet counts are not associated with bleeding risk among thrombocytopenic neonates. The *in vitro* bleeding time or CT-ADP, in contrast, which incorporates multiple factors contributing to primary hemostasis in whole blood, is strongly associated with bleeding scores among thrombocytopenic preterm neonates with gestational age <27 weeks.
- Additionally, these extremely preterm neonates also had a higher frequency of moderate-to-severe (grade 2-4) bleeding than more mature infants. While the reasons for their high bleeding risk are multifactorial, and likely include *in vivo* factors, 88% of infants with grades 3-4 bleeding had maximally prolonged CT-ADPs, suggesting that inadequate hemostasis might contribute to severe bleeding.
- Change in bleeding score showed a moderate positive correlation with the concurrent change in *in vitro* bleeding time (CT-ADP), but not with change in platelet count. Specifically, a decrease in CT-ADP (or improved bleeding time) in a given patient almost never coincided with an increase in the bleeding score (or more severe bleeding).
- The predictive value of CT-ADP for the next day's major-to-severe bleeding was less clinically significant compared with for the same day. Notably, the etiology of major-to-severe (grade 3-4) bleedings in our patient population was heterogenous, however, all of them revealed acute illnesses.

- Provided that our findings can be replicated and further clarified in larger cohorts, implementing CT-ADP into clinical practice may lead to more effective approaches of using platelt transfusions for thrombocytopenic preterm neonates.
- *Study 3* compared the *in vitro* responses of human neonatal vs. adult megakaryocyte (MK) progenitors to TPO, and two TPO mimetics: ROM and ELT. Compared to adult peripheral blood (PB, adult) progenitors, we found that umbilical cord blood (CB, neonatal) progenitors were hyperproliferative in response to both TPO mimetics, and generated low-ploidy yet fully-mature MKs.
- TPO and TPO mimetic concentrations determined the number of MKs generated in culture, but did not influence their maturation or ploidy level. ELT generated fewer MKs than TPO or ROM, and concentrations >10 μM did not support MK proliferation, similarly to two iron chelators (deferoxamine, deferiprone).
- These high ELT concentrations were also associated with evidence of intracellular iron depletion, and could be reversed by iron supplementation.
- At low concentrations (6μM), ELT stimulated megakaryopoiesis, but at higher concentrations (30μM), its iron-chelating effects predominated, and lead to reduced MK differentiation and proliferation.
- These findings may have implications when considering the use of TPO mimetics in neonates.

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