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Trends in mortality related to pulmonary embolism in the European Region, 2000-15: analysis of vital registration data from the WHO Mortality Database

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Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates

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Abstract

Plasma thrombopoietin (Tpo) levels distinguish thrombocytopenia resulting from increased platelet destruction or decreased platelet production. We investigated whether measuring plasma Tpo levels in thrombocytopenic newborns is of diagnostic value to establish the underlying mechanism of thrombocytopenia.

Tpo levels were measured with in-house developed ELISA in samples referred to our center because of thrombocytopenia noticed in the first 10 days of life. Clinical data were collected. Plasma Tpo levels <128 AU/ml were found in the majority (92%) of 121 newborns with immune-mediated thrombocytopenia ($n = 104$) and thrombocytopenia due to bacterial infections ($n = 7$); increased plasma Tpo levels (≥ 128 AU/ml) were found in thrombocytopenic newborns with severe asphyxia ($n = 24$). Highly increased plasma Tpo levels (>200 AU/ml) were found in thrombocytopenic neonates with congenital viral infections ($n = 22$) or amegakaryocytosis ($n = 6$). A plasma Tpo level <128 AU/ml excludes (negative predictive value 96%, 95% CI 90–99) severe asphyxia, congenital viral infections and amegakaryocytosis as the cause for thrombocytopenia in newborns.

Increased plasma Tpo levels indicate that thrombocytopenia in newborns, as a result of various nonimmune disorders, is often caused by (temporary) bone marrow suppression/failure. Measurement of plasma Tpo levels provides the clinician with an additional tool to decide on the differential diagnosis, the necessity for subsequent diagnostics and treatment in neonates with thrombocytopenia.

Introduction

In the neonatal intensive care unit, thrombocytopenia is one of the most common hematological problems [1–3]. The most significant risk of severe thrombocytopenia is intracranial hemorrhage, causing neurological impairment or even death. Early recognition of the underlying pathology guides appropriate treatment and is essential to predict the clinical course. However, the main etiologies and patterns of thrombocytopenia in neonates are highly variable and difficult to diagnose [1–3]. Therefore, we decided to include plasma Tpo level measurements in our routine diagnostic laboratory workup for suspected fetal and neonatal alloimmune thrombocytopenia (FNAIT).

Regulation of platelet production strongly depends on thrombopoietin (Tpo) levels. Tpo, mainly produced in the liver, binds to the c-mpl-receptors on CD34⁺ stem cells, megakaryocytes and platelets [4,5]. A sufficient megakaryocyte and platelet mass will passively eliminate free Tpo from the circulation [4–10]. Furthermore, Grozovsky et al. recently showed active regulation of Tpo levels via the hepatic expression of Tpo mRNA and

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protein regulation induced by the binding of desialylated (senescent) platelets to the hepatic Ashwell–Morell receptor (AMR) [11]. In previous studies, we showed that plasma Tpo levels are useful to discriminate thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) from thrombocytopenia caused by elevated platelet destruction as in immune thrombocytopenia (ITP) and FNAIT (normal or only slightly elevated Tpo levels) [12,13]. We have now analyzed the collected data to determine which underlying causes of neonatal thrombocytopenia show elevated plasma Tpo levels and investigated whether plasma Tpo levels can indeed help the clinician in early recognition of the underlying cause for the neonatal thrombocytopenia.

Patients and Methods

Between 1998 and 2013, plasma Tpo levels were measured in samples from 798 thrombocytopenic newborns, for whom blood samples were sent to our platelet and leukocyte serology reference laboratory. Clinical data, sent to our laboratory by the treating physicians as part of the diagnostic laboratory request, included the neonatal platelet count, gestational age at birth, birth weight, 1-min and 5-min Apgar scores, data on the presence of laboratory and clinical signs for perinatal asphyxia, bacterial or viral

infections or other early neonatal causes for thrombocytopenia, neonatal treatment and course in neonatal platelet count. All thrombocytopenic (platelet count $< 120 \times 10^9/L$) neonates for whom sufficient clinical information was available and material was sent to our laboratory within 10 days after birth were included in the study.

We compared the results of the neonatal plasma Tpo levels with a control group of healthy full-term neonates ($n = 51$, plasma Tpo levels measured in a previous study [13]), and we used a plasma Tpo cutoff level of 97 AU, being the highest level in a previously described FNAIT cohort for comparison of laboratory findings and therapeutic interventions between neonates with low or high plasma Tpo results [13].

A solid-phase sandwich ELISA for measurement of plasma Tpo concentrations was performed in real time with each patient studied as previously described [14]. One AU equals 9 pg of recombinant Tpo (Research Diagnostics, Flanders, NJ, USA).

Statistical analysis was performed with GraphPad Prism, version 6.04 (GraphPad Software, San Diego, CA, USA). For comparison of continuous values, the Mann–Whitney U test was used. For comparison of categorical variables, the Pearson's chi-square test or Fisher's exact probability test was used.

Results

For 585 of 798 referred neonatal samples, plasma Tpo levels were measured within 10 days postpartum. Sufficient clinical and laboratory data could be obtained for 303 thrombocytopenic neonates (supplemental Figure 1).

In our series of 303 included thrombocytopenic neonates, 137 neonates showed plasma Tpo levels ≤ 97 AU/mL and 166 showed plasma Tpo levels > 97 AU/mL.

Parity, gestational age, birth weight, platelet counts, hemoglobin level, leukocyte counts and day of blood collection were not significantly different for neonates with plasma Tpo levels ≤ 97 AU/mL and those with levels > 97 AU/mL (Table I). Only the 1- and 5-min Apgar scores were significantly lower in the 'high Tpo' group ($p < 0.001$ and $p = 0.004$, respectively). Neonatal treatment differed only regarding the use of immunoglobulins (IVIg). The latter was prescribed for 18 (13%) neonates with low plasma Tpo levels compared to 6 (4%) in the 'increased Tpo' group ($p = 0.002$). The majority of IVIg-treated neonates were categorized as suffering from FNAIT or maternal ITP (Table II).

Fetal/neonatal alloimmune thrombocytopenia was diagnosed as the main cause for the thrombocytopenia in 88 neonates. Twenty-eight of 88 (32%) FNAIT cases showed plasma Tpo levels > 97 AU/mL (Figure 1). A significant difference ($p < 0.0001$) was seen with the healthy controls (Table III).

Plasma Tpo levels did not significantly differ between the 'maternal ITP' cases ($n = 16$), diagnosed according to the guidelines of the American Society of Hematology, and the FNAIT cases ($p = 0.479$).

Acquired bacterial infection was clinically diagnosed for seven neonates based on predictive maternal problems (e.g., prolonged time of membranes rupture), neonatal behavior (e.g., quiet, nutritional problems), physical examination (fever, hypotonia), laboratory parameters (including cell count, leukocyte differentiation, CRP), and response on antibiotics treatment and by excluding other causes for thrombocytopenia. For none of the children, a positive culture was obtained and at the time of blood collection, between 3 and 7 day's postpartum, none of the children suffered from disseminated intravascular coagulation. Plasma Tpo levels were slightly increased ($p = 0.01$) compared to healthy controls but comparable ($p = 0.638$) with plasma Tpo levels in FNAIT. Plasma Tpo levels in low birth weight, i.e., small for

Table I. Comparison between the 'low' plasma Tpo and 'high' plasma Tpo group.

	Neonatal plasma Tpo level < 97 AU/mL	Neonatal plasma Tpo level ≥ 97 AU/mL	p Value*
	$n = 137$	$n = 166$	
Parity			
Nullipara	55 (40%)	66 (40%)	$p = 1.0$
Gestational age			
< 32 weeks	3 (1%)	9 (6%)	$p = 0.151$
32–36 weeks	17 (12%)	36 (22%)	$p = 0.034$
37–42 weeks	117 (86%)	118 (71%)	$p = 0.020$
> 42 weeks	0 (0%)	3 (2%)	$p = 0.07$
Range	24–42	25–43	
Neonatal birth weight (g)			
Median	3058	2827	$p = 0.004$
Range	760–4800	650–5500	
AS 1 min			
Median	8	6	$p < 0.001$
Range	1–10	1–10	
AS 5 min			
Median	9	8	$p < 0.001$
Range	5–10	2–10	
Sample drawn for Tpo measurement (days after birth)			
Median	4.0	3.9	$p = 0.679$
Range	0–10	0–10	
Platelet count ($\times 10^9/L$)			
Median	44	36	$p = 0.513$
Range	3–119	1–118	
Leukocyte count ($\times 10^9/L$)			
Median	16.1	15.8	$p = 0.05$
Range	3.9–34.1	3.9–36.9	
Hb (mmol/L)			
Median	11.6	11.2	$p = 0.045$
Range	6.5–15.2	4.4–15.5	

*Continuous variables are tested with Student's t -test or Mann–Whitney U test.

Categorical variables are tested with the Pearson chi-square test.

Sd: standard deviation; AS: Apgar scores.

gestational age (SGA, birth weight below the 10th percentile for gestational age, $n = 33$) and premature (born < 37 weeks gestational age, $n = 62$) thrombocytopenic neonates were significantly higher compared with both healthy controls and FNAIT cases. Furthermore, a significant inversed correlation ($p = 0.016$) was detected between birth weight and plasma Tpo levels. However,

Table II. Neonatal therapy.

	$n/137 < 97AU/mL$	$n/166 \geq 97AU/mL$	p Value*
Random platelet transfusion	38 (28%)	52 (31%)	$p = 0.498^*$
HPA-1a-negative platelet transfusion	25 (18%)	23 (14%)	$p = 0.296^*$
IVIg treatment	18 (13%)	6 (4%)	$P = 0.002^*$
Antibiotics	10 (7%)	18 (11%)	$p = 0.290^*$
RBC transfusion	0 (0%)	4 (2%)	$p = 0.129^\dagger$
RBC exchange transfusion	1 (1%)	2 (1%)	$p = 1.0^\dagger$
No treatment	72 (53%)	78 (47%)	$p = 0.335^*$

*Pearson chi-square test, † Fisher's exact probability test.

HPA: human platelet antigen; IVIg; intravenous immunoglobulin; RBC, red blood cell.

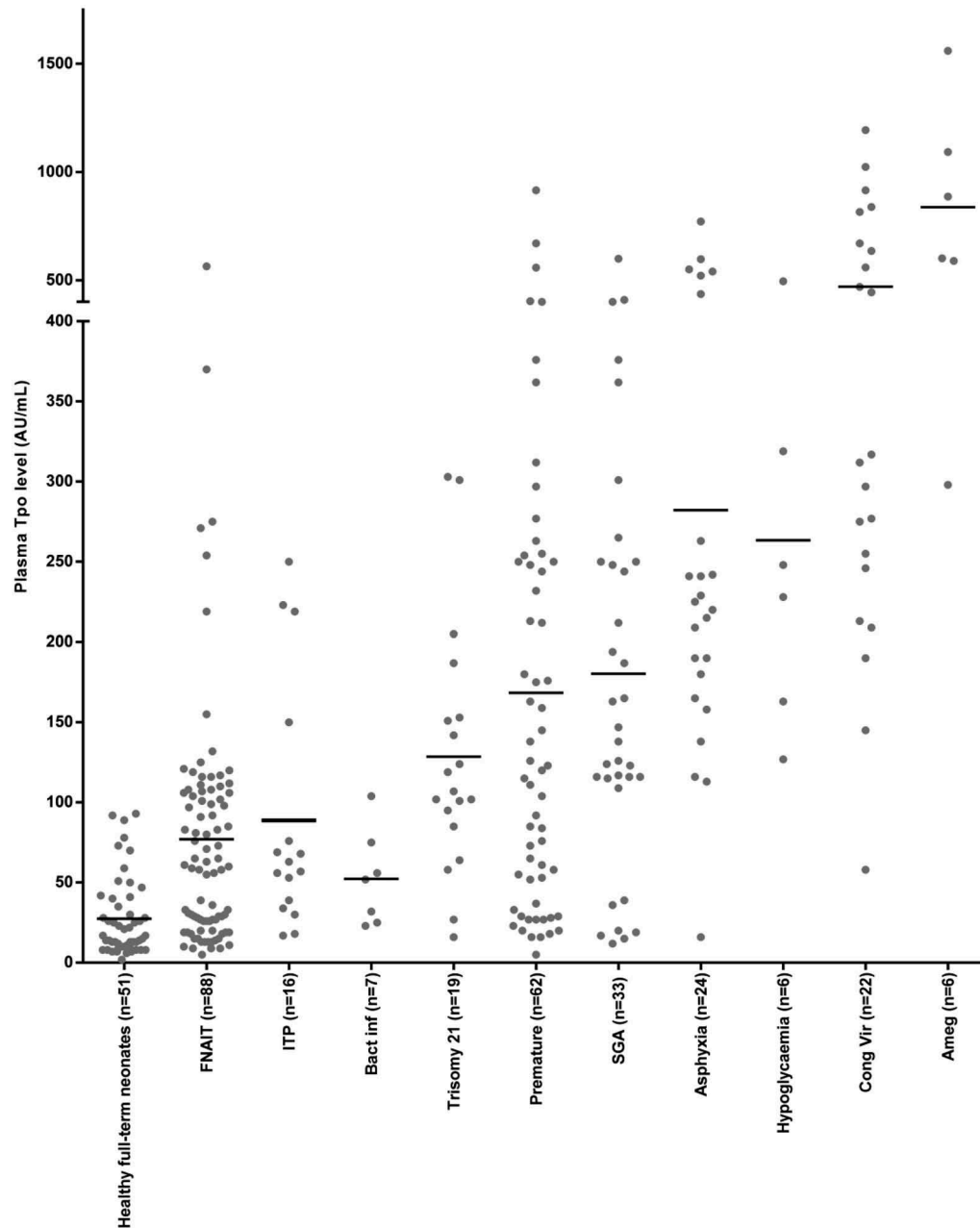


Figure 1. Plasma Tpo values for the various neonatal disorders.

Table III. Platelet count and plasma Tpo level parameters.

	n	Platelet count ($\times 10^9/L$) Median (range)	Plasma Tpo (AU/mL) Median (range)	Mann–Whitney <i>U</i> test	
				Tpo levels compared to healthy controls <i>p</i> =	Tpo levels compared to FNAIT <i>p</i> =
Healthy full-term neonates ^a	51	Not tested	17 (2–93)		
FNAIT	88	13 (5–117)	60 (5–565)	<0.0001	
ITP	16	19 (8–103)	60 (17–250)	<0.0001	0.479
Prematurity	62	40 (2–116)	113 (5–916)	<0.0001	<0.0001
Small for gestational age	33	50 (5–118)	143 (15–600)	<0.0001	<0.0001
Asphyxia	24	45 (2–110)	220 (16–772)	<0.0001	<0.0001
Cong viral infections	22	30 (7–97)	314 (118–1194)	<0.0001	<0.0001
Trisomy 21	19	50 (15–84)	107 (16–303)	<0.0001	<0.0001
Bacterial infections	7	50 (12–119)	52 (23–104)	0.01	0.638
Amegakaryocytosis	6	20 (2–43)	745 (298–1560)	<0.0001	<0.0001

^aPlasma Tpo levels measured in a previous study [13].

Tpo, thrombopoietin; FNAIT: fetal/neonatal alloimmune thrombocytopenia; ITP: immune thrombocytopenia.

most cases were combined with other pathology, and for that reason, the precise contribution of SGA and prematurity cannot be identified properly.

In our series, congenital viral infections ($n = 22$), next to amegakaryocytosis, showed the highest plasma Tpo levels. All, but one, (95%) neonates suffering from (suspected) congenital viral infections showed highly elevated Tpo levels. Maternal and neonatal viral serology, viral cultures and/or viral PCR tests showed; 14 recent cytomegalovirus (CMV) infections including one twin pregnancy, one herpes simplex virus infection and one parvovirus infection. For five neonates, congenital viral infections were supported by hepatosplenomegaly, abnormal blood counts, hemolytic anemia, elevated transaminases and elevated serum bilirubin levels, but viral diagnostics were not or only partly performed. One neonate, born after 36 weeks of gestation, birth weight 2840 g, platelet count $23 \times 10^9/L$ and plasma Tpo level of 58 AU/mL, did not show any signs of infection; however, viral serology showed CMV antibodies of the IgG and IgM class. As no other cause for the thrombocytopenia could be detected, congenital CMV infection was accepted as most probable cause. The proven or suspected viral infections were combined with prematurity in 8 neonates (including the twin neonates), leaving 14 neonates without multifactorial causes for the elevated plasma Tpo levels.

Increased plasma Tpo levels were measured in all, but one, neonates with asphyxia ($n = 24$), as diagnosed by Apgar scores (<4 after 1 min), cord blood pH (≤ 7.05) and/or base deficit and necessary resuscitative interventions. One neonate, born after emergency caesarean section was carried out for fetal distress at 37 weeks gestational age, with Apgar scores 3 and 8 after 1 and 5 min, cord blood pH 7.05 and platelet count $66 \times 10^9/L$ at day 4 postpartum, showed a plasma Tpo level of 16 AU/mL.

As shown previously [14], highly elevated Tpo levels (>200 AU/mL) were found in the group with thrombocytopenia due to bone marrow failure ($n = 6$); i.e., bone marrow biopsy showed a substantially reduced number or absence of megakaryocytes in all these six cases. Furthermore, in these cases, mutations in the coding-regions of the *c-mpl* gene, encoding for the Tpo-receptor, were shown, leading to an amino-acid substitution or to a premature stop-codon.

Although, neonatal hypoglycemia can be due to various clinical underlying causes and on itself is not associated with thrombocytopenia, significantly elevated plasma Tpo levels were measured in six neonatal hypoglycemia (plasma glucose levels <30 mg/dL) cases. Two of those neonates were born with congenital cardiac defects of whom one was also premature and SGA (36 weeks, 1515 g) and the other premature (36 weeks, 2940 g), two with extreme birth weights of 4400 and 5500 g as a result of maternal pregnancy diabetes, one after 43 weeks pregnancy by caesarean section and one neonate suffering from convulsions e.c.i.

Trisomy 21 was diagnosed for 19 neonates. In this group, platelet counts and plasma Tpo levels showed a significant inversed correlation ($p = 0.001$, data not shown). Neonates with platelet counts $<50 \times 10^9/L$ all produced high plasma Tpo levels.

Miscellaneous neonatal pathology was seen for 35 cases, i.e., fetal/neonatal intracranial hemorrhage e.c.i. ($n = 10$), red blood cell antagonisms ($n = 5$), maternal preeclampsia ($n = 12$) and rare syndromes ($n = 8$). Elevated or highly elevated plasma Tpo levels were produced for all syndromal cases, i.e., hygroma colli, Jacobson syndrome, Langerhans cell histiocytoma, thrombocytopenia absent radius, Noonan syndrome, Fragile X syndrome, with the exception of a neonate suffering from Turner's syndrome and a mild thrombocytopenia (platelet count $88 \times 10^9/L$).

Despite extensive clinical and laboratory investigation, no clear explanation for the thrombocytopenia could be detected for 33 neonates, with plasma Tpo levels ranging from 15 to 1015 AU/mL (mean 194 AU/mL, Std 265 AU/mL).

Discussion

The neonatal samples were drawn between day 1 and 10 postpartum. Although platelet counts for all neonates included in this study were below $120 \times 10^9/L$, it might be that with increasing platelet counts in the first day's postpartum, possibly due to treatment, plasma Tpo levels inversely decreased. However, despite this uncertainty, the difference between the Tpo levels in immune- and nonimmune-mediated thrombocytopenia is clear and does not seem to be dependent on which day the samples were drawn. Plasma Tpo levels in the fetus and newborns with thrombocytopenia depend on the production of Tpo in the more or less mature liver and the scavenging of Tpo by binding to the c-mpl receptors, expressed on platelets, platelet precursors and most early hematopoietic progenitor cells [15,16]. Tpo levels can be decreased by increased numbers of blast cells expressing c-mpl binding free Tpo, which may occur in myeloproliferative disorders in newborns with Trisomy 21. In the series of newborns with Trisomy 21 ($n = 19$), no transient myeloproliferative disorders were reported. As also described by Matsubara et al. [17], we detected for newborns with Trisomy 21, a significant inversed correlation ($p = 0.001$) between platelet counts and Tpo levels. This indicates again that the concentration of free circulating Tpo in blood depends largely on the number of megakaryocytes in the bone marrow and platelets in the peripheral blood, suggesting a temporary impaired platelet production.

Confirming previous studies, we observed normal or slightly elevated plasma Tpo levels for thrombocytopenic neonates with antibody-mediated fetal/neonatal platelet destruction disorders, as seen for FNAIT [13]. It is intriguing that 6/104 (6%) neonates with a primary diagnosis FNAIT or maternal ITP showed highly (>200 AU/mL) increased plasma Tpo levels. It may well be that in spite of extensive investigations and the lack of accompanying clinical signs, an additional mechanism for the thrombocytopenia was missed. Another explanation could be that these neonates temporarily suffered from exhausted platelet production due to limited ability of neonatal megakaryocytes to increase their size in response to increased platelet demand, but unfortunately, no bone marrow investigations were performed [18,19]. Antibody-mediated platelet destruction occurs mainly in the spleen, but it is recently suggested that glycoprotein Ib/IX-specific (auto-)antibodies can cause glycoprotein desialylation, resulting in an alternative route for platelet destruction by binding to the AMR, leading to increased Tpo

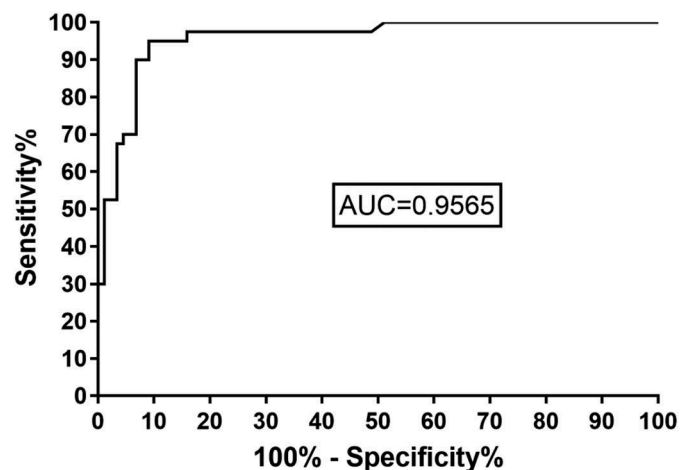


Figure 2. ROC-curve plasma Tpo levels for thrombocytopenic neonates due to FNAIT ($n = 77$) and ITP ($n = 14$) compared with congenital viral infections ($n = 14$), asphyxia ($n = 24$) and amegakaryocytosis ($n = 6$) (only cases without multifactorial causes are included). The optimal sensitivity of 95% (95% CI 83.1–99.4) and specificity of 91% (95% CI 82.9–96) combination was shown at a plasma Tpo level of 128 AU/mL.

production [11]. We did not detect GPIb-specific (auto-)antibodies in the 'FNAIT' or 'ITP' neonates with increased Tpo levels, and therefore, this cannot explain our findings.

Normal plasma Tpo levels were detected in the seven suspected (for none of these neonates, a positive blood culture was detected) bacterial infection cases indicating an increased platelet destruction. This notion is supported by Oygur et al. [20], who found that the principal mechanism responsible for thrombocytopenia in bacterial (or fungal) infections in neonates seemed to be accelerated platelet destruction, probably secondary to endothelial damage with subsequent platelet adhesion and aggregation. Alternatively, bacterial infections may lead to platelet lysis or removal by the reticuloendothelial system.

Elevated to highly elevated plasma Tpo levels were detected in the majority of neonates diagnosed with congenital viral infections,

severe asphyxia and amegakaryocytosis (Table III, Figure 1). The increased plasma Tpo levels can be the result of disruption of the myeloproliferative leukemia protein (c-mpl) receptor function due to mutations in the *c-MPL* gene, resulting in congenital amegakaryocytic thrombocytopenia, impaired megakaryocytopoiesis due to immaturity of the bone marrow function, progenitor and/or non-progenitor bone marrow cell damage possibly caused by active viral infections (CMV, Parvo, Herpes, HIV, Rubella) or oxygen deprivation and as a result of increased cortisol levels after stress causing reduced c-mpl expression on the cell membranes [18,19,21,22].

If used in a diagnostic setting to distinguish immune-mediated neonatal thrombocytopenia (FNAIT and ITP) from (temporary) platelet production failure (asphyxia, congenital viral infection and amegakaryocytosis), the area under the curve is 0.957 (95% CI 0.922–0.991).

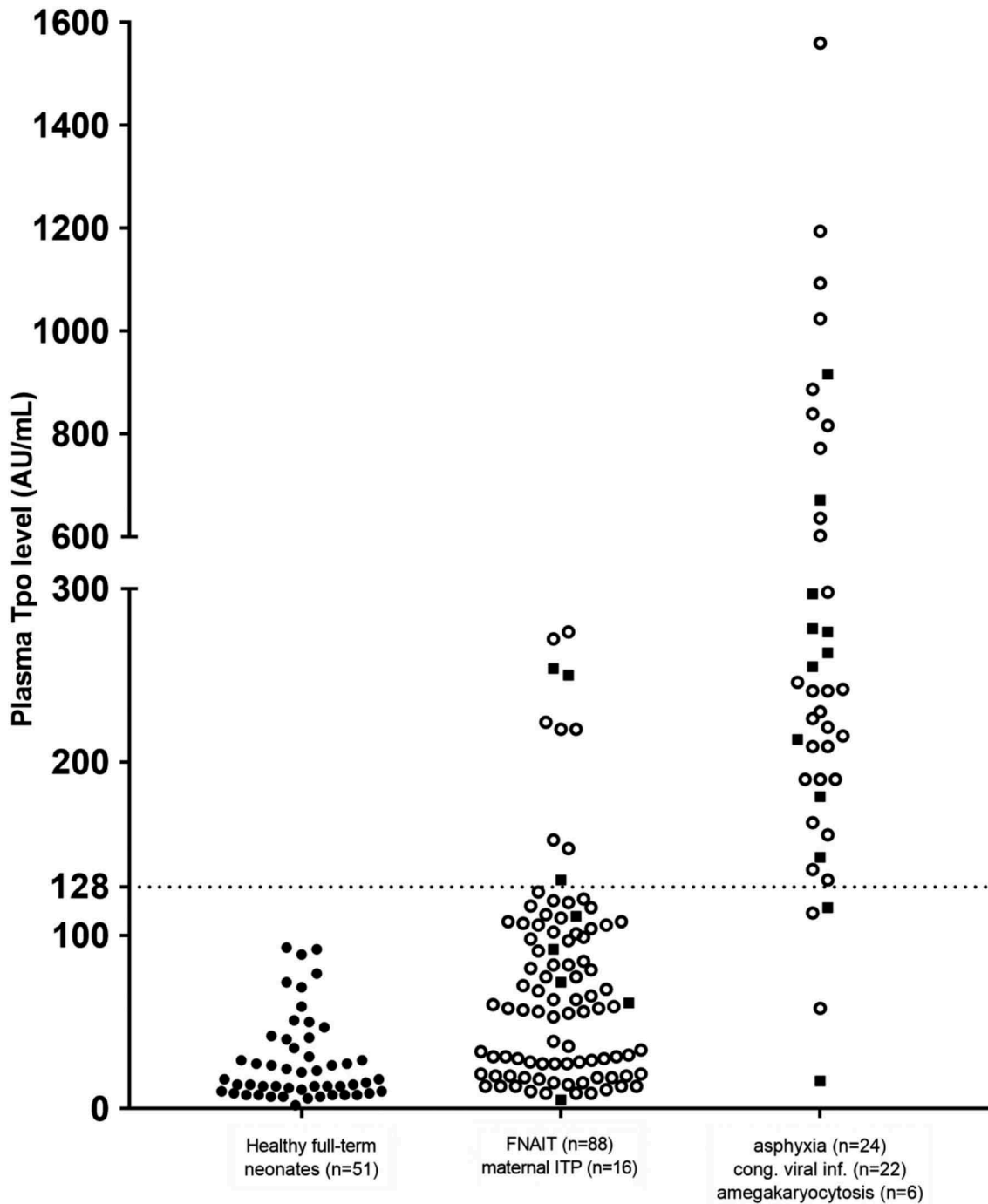


Figure 3. Tpo levels in neonates with different causes for thrombocytopenia. Open circles are used for those cases without secondary pathology (FNAIT $n = 77$, ITP $n = 14$, asphyxia $n = 21$, congenital viral infection $n = 14$, amegakaryocytosis $n = 6$).

The receiver operating characteristic (ROC)-curve shows an optimal sensitivity of 95% (95% CI 83.1–99.4) and specificity of 91% (95% CI 82.9–96) combination at a plasma Tpo level of 128 AU/mL (Figure 2).

In our series, only 4 (8%) of 52 neonates categorized as suffering from severe asphyxia, congenital viral infections or amegakaryocytosis showed a plasma Tpo level <128 AU/mL. On the other hand, only 12 (12%) of 104 neonates suffering from FNAIT and maternal ITP and bacterial infections showed plasma Tpo levels \geq 128 AU/mL (Figure 3). Subsequently, we can use plasma Tpo levels as an additional diagnostic tool, wherein the measuring of a neonatal plasma Tpo level < 128 AU/mL excludes (negative predictive value 96%, 95% CI 90–99) severe asphyxia, amegakaryocytosis and congenital viral infections as causes for neonatal thrombocytopenia. This can assist the treating physician in deciding on any subsequent treatments and/or the necessity for further diagnostic investigations (e.g., the necessity for diagnosing FNAIT). In this study, we did not look at the neonatal mean platelet values and immature platelet counts, which may also contribute to the elucidation of the underlying cause for thrombocytopenia. Further studies can focus on the comparison of these values and plasma Tpo levels.

We conclude that determination of plasma Tpo levels in thrombocytopenic newborns can be used as one of the diagnostic parameters to guide the differential diagnosis and most optimal treatment.

Declaration of Interest

The authors report no declaration of interest.

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Statement of Contribution

Dr Porcelijn conceptualized and designed the study, carried the acquisition of data and the initial analysis, drafted the initial manuscript and reviewed, revised and finally approved the manuscript. Dr Porcelijn agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ms Huiskes and Ms Onderwater designed the data collection instruments and collected data, reviewed, revised and finally approved the manuscript. Ms Huiskes and Ms Onderwater agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Dr Folman designed the data collection instruments, analyzed and interpreted the data, reviewed, revised and finally approved the manuscript. Dr Folman agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Professor Zwaginga, and Professor de Haas supervised data collection, analyzed and interpreted the data, critically reviewed the manuscript for important intellectual content and finally approved the manuscript. Professor Zwaginga and Professor de Haas agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Supplementary Material

Supplemental data for this article can be accessed [here](#).

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