Immature platelet values indicate impaired megakaryopoietic activity in neonatal early-onset thrombocytopenia

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Summary

Newly released platelets, referred to as immature platelets, can be reliably quantified based on their RNA content by flow cytometry in an automated blood analyser. The absolute number of immature platelets (IPF#) and the immature platelet fraction (IPF%) reflect megakaryopoietic activity. We aimed to analyse the implication of these parameters in analysing the pathomechanism of early-onset neonatal thrombocytopenia. Platelet counts and IPF were determined at day 1 to 3 (d1 to d3) in 857 neonates admitted to intensive care. In thrombocytopenic patients (platelet counts<150 x 10⁹/l, n=129), IPF# was significantly lower (8.5 ± 2.7 x 10⁹/l) than in non-thrombocytopenic neonates (9.5 ± 3.6 x 10⁹/l, n=682, p<0.05). IPF% was significantly higher in thrombocytopenic (9.3 ± 7.9%) vs. non-thrombocytopenic neonates (4.1 ± 1.8%, p<0.001). While neonates with early-onset infection (n=134) had lower platelet counts (199 ± 75 x 10⁹/l) compared to controls (230 ± 68 x 10⁹/l, n=574, p<0.01), there were no differences in IPF# or IPF%. Likewise, “small for gestational age” infants (SGA, n=149) had lower platelet counts at d1 (199 ± 75 x 10⁹/l, p<0.001) than controls, but no differences in IPF. A trend towards lower IPF# was detected if SGA infants with platelet counts <100 x 10⁹/l (5.4 ± 3.9 x 10⁹/l, n=11) and thrombocytopenic neonates with infection (9.9 ± 7.3 x 10⁹/l, n=10, p=0.11) were compared. The evaluation of IPF# indicates that thrombocytopenia in neonates is likely due to a combination of increased platelet consumption and inadequate megakaryopoietic response by the neonatal bone marrow. Furthermore, SGA neonates with moderate and severe thrombocytopenia might have a pronounced suppression of megakaryopoiesis compared to neonates with infection.

Keywords
Immatre platelet count, reticulated platelets

Introduction

Thrombocytopenia, defined by platelet counts <150 x 10⁹/l, is one of the most common haematological problems in preterm and term neonates, because it affects 18% to 35% of all patients admitted to neonatal intensive care units (NICU) (1, 2). In extremely low birth weight neonates (birth weight <1,000 g) thrombocytopenia occurs at a rate more than twice that reported among the general NICU population (3). Among thrombocytopenic extremely low birth weight infants, almost 40% suffer from severe thrombocytopenia as defined by platelet counts <50 x 10⁹/l (3). In preterm infants, early-onset thrombocytopenia is often caused by chronic hypoxia (associated with placental insufficiency, intrauterine growth retardation) and is characterised by moderate reduction of platelets (50–100 x 10⁹/l) which recover spontaneously during the first week after birth. Bacterial infection also causes early-onset thrombocytopenia, which is characteristically severe (platelet counts <50 x 10⁹/l) and associated with significant risk of haemorrhage both in preterm and term neonates (4). Since both chronic hypoxia and acute inflammation can occur simultaneously, particularly in preterm neonates, the exact pathomechanism of early-onset thrombocytopenia remains unclear in half the cases. This often makes decisions on further diagnostics and treatment difficult.

Thus, a routine non-invasive laboratory test that allows evaluation of the megakaryopoietic activity and amount of platelet production would be useful, in particular if it distinguishes between inappropriate platelet production and increased platelet consumption. A first step towards an improved diagnostic marker was taken almost 40 years ago, when Ingram and Coopersmith (5) demonstrated that induction of thrombocytopenia by bleeding results in the release of RNA-containing platelets which are bigger in size and more reactive than mature platelets. Analogous to reticulocytes, these platelets have been named “reticulated” platelets. Evidence indicates that the number of reticulated platelets correlates with the megakaryopoietic activity in the neonatal and adult bone...
marrow (6, 7). Reticulated platelets can be counted by fluorescence activated cell sorting (FACS) after adding fluorescent agents that bind to the RNA of platelets. Further technical developments have improved the ability to accurately distinguish reticulated platelets from reticulocytes and other cell types. Nowadays, the immature platelet fraction (IPF) can be routinely measured in fully automated haematology cell analysers (such as the XE-Pro Series, Sysmex, Kobe, Japan) without requirement of additional blood volume (8). Recent data on immature platelets in patients with acute coronary syndrome indicate that their increased haemostatic potential may contribute to thrombus formation and lead to clinical decision making on haemostatic treatment of adults (9). Previous studies have shown the value of IPF% for diagnosing the origin of thrombocytopenia and for clinical decision-making with regard to management of platelet transfusion strategies, e.g., after bone marrow transplantation (10–12) or in isolated thrombocytopenia (8, 13). In clinical neonatology the advantage of this novel method is to limit loss of blood volume, particular in preterm neonates. We recently demonstrated that measurement of IPF in neonates is feasible and established reference values for neonates (14).

The aim of this study was to investigate IPF# and IPF% as novel, routinely available laboratory parameters in early-onset neonatal thrombocytopenia and to test whether one of both parameters has a more reliable diagnostic value. We hypothesised that megakaryopoietic activity is a) inhibited in neonatal thrombocytopenia associated with intrauterine growth retardation and b) moderately increased in early-onset thrombocytopenia caused by infection (15–17).

**Methods**

Infants who were admitted to our NICU between January 2008 and March 2009 were prospectively enrolled in this study if they had blood count measurements within the first three days of life (d1, d2, d3). Furthermore, the attending physician decided to repeat complete blood count on d2 or d3 only as indicated by the clinical condition of the baby. Written parental informed consent and organisational approval were obtained. For data analysis, we excluded blood samples obtained after platelet transfusion, since transfused platelets may interfere with IPF values (18). Blood samples were collected either from indwelling arterial catheters or peripheral veins only for blood examination due to clinical indication to avoid additional loss of blood volume. Concentrations of interleukin-6 (IL-6) and/or C-reactive protein were determined by means of an automated immunoassay (IMMULITE 2000, Siemens, Erlangen, Germany; COBAS, Roche, Mannheim, Germany) for clinically indicated purposes. Early-onset infection was considered if the concentration of either interleukin-6 or C-reactive protein exceeded 100 pg/ml or 1 mg/dl, respectively. To clarify the implication of IPF in early-onset thrombocytopenia, infants were classified as small for gestational age (SGA) according to their birth weight below the 10th percentile or appropriate for gestational age if the birth weight was between the 10th and 90th percentile. A control group was defined consisting of appropriate for gestational age infants without infection as proven by normal IL-6 and C-reactive protein concentration.

**Immature platelet fraction**

At d1, d2, and/or d3 approximately 200 µl blood were collected into EDTA-containing tubes (Becton Dickinson, Franklin Lakes, NJ, USA). IPF and platelet counts were immediately analysed by the fully-automated haematological Sysmex XE-2100 analyser (Sysmex, Kobe, Japan) equipped with the XE-IPF-Master software. After incubation with a dye (Red Search II, Sysmex), RNA-containing cells (reticulocytes and RNA-containing platelets) were stained. Then blood specimens were passed through a laser diode beam, and the resulting forward scatter light and sideward fluorescence intensity was measured. A pre-set gate discriminated between mature and immature platelets. IPF were expressed as a percentage (IPF%) of the platelet count or as an absolute number (IPF#).

**Statistical analysis**

Patients’ characteristics are described as numbers and percentages or as median and range. The data on blood parameters are shown as mean ± SD. Individual differences in the blood parameters between the various days of life are expressed as means. Comparisons of birth weight and gestational age are performed with the Mann-Whitney-Wilcoxon test. Categorical variables (SGA; infection) are compared with the Fisher’s exact test. Patients are classified according to their degree of thrombocytopenia (severe thrombocytopenia: platelet counts <50×10^9/l; moderate thrombocytopenia: platelet counts 50–99×10^9/l; mild thrombocytopenia: platelet counts 100–149×10^9/l; no thrombocytopenia: platelet counts ≥150×10^9/l) (4). Comparisons of IPF% and IPF# between these groups are performed using ANOVA and Bonferroni’s multiple comparison procedure. Differences in blood cell parameters between the patient groups are tested by the paired t-test or unpaired t-test, as appropriate. Pearson’s correlation coefficient is calculated to investigate the relationship between gestational age and platelet counts, IPF% or IPF#. Statistical analysis is performed using the software Statgraphics (Version 5, Stat Point, Herndon, VA, USA). Statistical significance is defined as a p-value <0.05.

**Results**

Over a fifteen-month period, we analysed 1,517 blood counts from 857 newborns. Clinical characteristics are summarised in Table 1. The control group consisted of 574 neonates with a median birth weight of 2,832 g (650–4,500 g) and a median gestational age of 37.0
At d1 811 blood samples were measured, at d2 332 samples and d3 374 samples, respectively. One hundred twenty-nine patients (15%) were thrombocytopenic. To generate reference values for IPF# and IPF%, we analysed all neonates (n=682) with platelet counts >150 x 10⁹/l. The mean absolute IPF# in these neonates was 9.5 ± 3.8 x 10⁹/l and the mean IPF% was 4.1 ± 1.8%. Gestational age and platelet counts showed a statistically significant, but low correlation (r = 0.13, p<0.001). There was no statistically significant correlation between gestational age and IPF# or IPF%. As shown in Table 2, in thrombocytopenic neonates with platelet counts <150 x 10⁹/l there was a significantly lower birth weight (p<0.01), a lower mean IPF# (p<0.05) and higher IPF% (p<0.001) compared to non-thrombocytopenic neonates.

To simplify the interpretation of IPF% and IPF# in different thrombocytopenic conditions, we categorised the patients into four groups according to their platelet counts. With increasing severity of thrombocytopenia, the IPF% rose significantly (p<0.001). IPF% was more than three times higher in severe thrombocytopenia (platelet counts <50 x 10⁹/l) compared to non-thrombocytopenic patients (Table 2). However, the absolute number of immature platelets decreased in thrombocytopenic infants. Most notably, IPF# in neonates with severe thrombocytopenia (IPF# = 4.7 x 10⁹/l) and non-thrombocytopenic neonates (IPF# = 9.5 x 10⁹/l) showed statistically significant differences (p<0.001, Fig. 1B).

Regarding factors that may influence the platelet counts and megakaryopoietic activity, we also analysed platelet counts and IPF values in SGA infants vs. controls. The mean platelet counts at d1, d2, and d3 were always significantly lower in SGA infants than in

- **Table 2: Immature platelet fraction (mean ± SD) at day one and epidemiological data (median and range) in thrombocytopenic neonates vs. non-thrombocytopenic neonates.**

<table>
<thead>
<tr>
<th></th>
<th>Platelet counts ≥150 x 10⁹/l</th>
<th>Platelet counts &lt;150 x 10⁹/l</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPF# (x 10⁹/l)</td>
<td>9.5 ± 3.6</td>
<td>8.5 ± 2.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IPF (%)</td>
<td>4.1 ± 1.8</td>
<td>9.3 ± 7.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2580 (584 – 4840)</td>
<td>2215 (400 – 4550)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>37.0 (23.3 – 42.1)</td>
<td>35.6 (24.8 – 41.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>Infection, n (%)</td>
<td>100 (15%)</td>
<td>34 (24%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Small for gestational age, n (%)</td>
<td>109 (16%)</td>
<td>40 (34%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infection and small for gestational age, n (%)</td>
<td>10 (0.2%)</td>
<td>9 (7%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
controls. However, neither the absolute IPF# nor the IPF% differed significantly between SGA and controls (Fig. 2A). IPF# was lower when moderately and severely thrombocytopenic SGA (IPF# = 5.4 ± 3.9 x 10^9/l, n=11) infants and thrombocytopenic neonates with infection (IPF# = 9.9 ± 7.3 x 10^9/l, n=10) were compared, but this difference was not statistically significant (p=0.11, Fig. 3A and B). In the case of moderate thrombocytopenia, a trend towards lower IPF# (p=0.16) was noticed in SGA neonates compared to neonates with infection. This analysis is based only on a small number of patients because the majority of neonates presented a thrombocytopenia of a different, unclassified aetiology (Table 1).

In neonates with early-onset infection the mean platelet counts were significantly lower than in controls at d1 and d3 (Fig. 2B). Infants with early-onset infection were more frequently thrombocytopenic, especially if infection was combined with growth retardation (Table 2). To assess the megakaryopoietic activity in neonates with early-onset infection, we compared the absolute IPF# and IPF% with controls. As shown in Figure 2B, there was no statistically significant difference between the two groups.

In all neonates who had serial blood counts at d1 plus at d2/d3, the course of platelet counts and IPF were analysed. Pair-matched longitudinal data on platelet counts indicated a significant decrease in platelet counts from d1 (219 x 10^9/l) to d2 (191 x 10^9/l) or from d1 (219 x 10^9/l) to d3 (202 x 10^9/l, p<0.001). The value of IPF# decreased in parallel to the platelet counts (d1: 9.3 x 10^9/l; d2: 8.6 x 10^9/l; d3: 8.8 x 10^9/l; p<0.001), whereas the percentage of IPF increased in parallel (d1: 4.9%; d2: 6.1%; d3: 5.5%; p<0.001).
Discussion

Here we present the first study specifically considering the diagnostic values of the absolute number of IPF (IPF#) and the percentage of immature platelets (IPF%) in early-onset neonatal thrombocytopenia associated with neonatal infection and/or growth retardation. The IPF% was significantly higher in neonates with severe thrombocytopenia, but the absolute number of IPF was significantly lower. Since Kienast et al. (6) described thiazole orange-uptake to platelets as a marker for platelet RNA, there is ongoing debate whether percentages or absolute counts of reticulated platelets should be considered for estimating the megakaryopoietic activity. Kienast et al. reported on patients with thrombocytopenia caused by increased platelet destruction and patients with thrombocytopenia due to impaired platelet production. In the first group absolute numbers of thiazole orange-positive platelets were slightly decreased, whereas the percentage of thiazole orange-positive platelets was higher compared to controls. Thrombocytopenic patients with reduced numbers of bone marrow megakaryocytes had six-fold lower absolute numbers of thiazole orange-positive platelets compared to normal subjects. However, the percentage of thiazole orange-positive platelets did not differ from those of the control group (6). Furthermore, Briggs et al. reported similar results measuring IPF% and IPF# in adults with thrombocytopenia and controls. In normal adults IPF% was 3.4%, and IPF# 8.6 x 10⁹/l. In adults with severe immune thrombocytopenia, IPF% was increased (22.3%), but IPF# remained nearly unchanged (7.8 x 10⁹/l) (8). However, in neonates with severe thrombocytopenia IPF# (4.7 x 10⁹/l) totalled only half the number measured in controls (IPF# 9.5 x 10⁹/l, Fig. 1B). In neonates with severe thrombocytopenia IPF# was still remarkably higher compared to patients with bone marrow failure after chemotherapy (IPF# 0.7 x 10⁹/l), so that neonates with severe thrombocytopenia are able to produce six times the amount of immature platelets. These data and our results (Fig. 1B) indicate that the absolute IPF number should also be taken into account as a marker of megakaryopoietic activity, especially in patients with low platelet counts.

To date, only three studies have been published on the analysis of reticulated platelets by flow cytometry in neonates. The results of these studies conflicted partially, probably due to variations in methods and study design (7, 19, 20). As in our study, Saxonhouse et al. (19) and Joseph et al. (20) reported higher percentages of reticulated platelets in term and preterm neonates than in adults, whereas Peterec et al. (7) found higher percentages of reticulated platelets only in preterm neonates. These findings may reflect the overall higher megakaryopoietic activity in neonates, likely due to higher basal circulating Tpo concentrations in the foetus and the neonate compared to adults (17, 21–24). Saxonhouse et al. (19) described a correlation between percentages of reticulated platelets and gestational age in twenty non-thrombocytopenic infants but did not consider IPF#.

During the first three days after birth, IPF# was lower in thrombocytopenic vs. non-thrombocytopenic neonates (Fig. 1B). When discriminative degrees of thrombocytopenia were taken into account, this difference became significant in the groups of neonates with platelet counts <50 x 10⁹/l and with platelet counts 50–99 x 10⁹/l.

The moderate decline of the platelet counts over the first three days was partially compensated by an increase in IPF%, but not resulting in maintenance of absolute IPF# in severe thrombocytopenia (Fig. 1A). This suggests a temporarily slight depression in megakaryopoietic activity, which is in line with previous data on the number of circulating megakaryopoietic progenitor cells (16). Even in neonates without haematological disorders, the number of circulating megakaryopoietic cells declined during the first days of life, and the number of megakaryopoietic progenitors was more reduced in thrombocytopenic vs. non-thrombocytopenic neonates (25). The combined data indicate that platelet numbers decline within the first days after birth as a result of a temporarily slight reduction in megakaryopoietic activity.

IPF can be reliably measured using standardised FACS technology in peripheral blood samples with high reproducibility, precision and stability (8, 14). These IPF measurements are readily available without further processing of the samples. We recently provided evidence that in non-thrombocytopenic neonates IPF# values were higher than reported in adults and that IPF predicts the course of neonatal thrombocytopenia (14).

We could not find any significant differences in the IPF value between SGA and appropriate for gestational age infants or be-
tween neonates with and without infection. Interestingly, during moderate or severe thrombocytopenia a trend towards lower IPF# in SGA compared to neonates with infections was detected. However, the number of patients with severe and moderate thrombocytopenia included in this analysis (Fig. 3A) was extremely small, so it is possible that a difference was not detected because of insufficient sample size in those groups. The IPF count in thrombocytopenic SGA neonates may reflect a slightly fetal decreased megakaryopoiesis, which could only partially compensate a high demand of platelets in favour of an enhanced hypoxia-induced erythropoiesis (26). In line with this finding, Sola et al. (17) described in thrombocytopenic SGA neonates inadequate low circulating Tpo concentrations despite a reduced megakaryopoietic mass in bone marrow specimens analysed within the first 42 days after birth. They concluded that these SGA infants suffer from a hypogenerative megakaryopoiesis due to impaired Tpo production. We propose that measurements of IPF values combined with the measurement of circulating Tpo plasma concentrations may be an efficient approach to optimise the management of neonatal thrombocytopenia of unclear origin, at least initially without invasive diagnostics such as bone marrow smears.

In conclusion, measurement of IPF is a valuable parameter for evaluating megakaryopoietic activity in peripheral blood due to its reliability, convenient application and availability around the clock. The evaluation of IPF# indicates that moderate or severe thrombocytopenia in neonates is likely due to a combination of increased platelet demand and an inadequate megakaryopoietic response by the neonatal bone marrow. Furthermore, SGA neonates with moderate and severe thrombocytopenia might have a pronounced suppression of megakaryopoiesis compared to neonates with infection.

References