Original Articles

Title: Immature platelet fraction and its kinetics in neonates

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Abstract

Thrombocytopenia is a common abnormality encountered in the neonatal period, and immature platelet fraction (IPF) may be an informative indicator of thrombopoiesis; however, data on IPF in neonates are scarce. To define reference intervals (RIs) and factors affecting IPF in neonates, we measured IPF of 533 consecutive neonates. With a multiple regression analysis of 330 newborns with normal platelet counts at birth, premature delivery, neonatal asphyxia, intrauterine infection, chromosomal abnormalities and respiratory disorders were identified as independent factors for IPF%. The RIs of IPF% and absolute IPF value (A-IPF) in neonates were determined to be 1.3%-5.7% and 3.2-14.5×10^9/L, respectively. On day 14 after birth, IPF% increased to twice the value at birth and thereafter returned to the previous value on day 28. Reticulocyte counts, in contrast, were the lowest at day 14. IPF% was increased in 16 thrombocytopenic patients with various clinical conditions, especially those with immune-mediated thrombocytopenia. IPF in neonates may be evaluated essentially based on the same RIs as in adults, although some precautions must be taken when evaluating IPF in neonates in the first two weeks of life. IPF may be useful for evaluating thrombopoiesis and thrombocytopenia in neonates.

Key words

Reticulated platelet, Immature platelet fraction, Neonate, Thrombocytopenia, Thrombopoiesis

Introduction

Platelets just released from megakaryocytes, containing abundant RNA, are called reticulated platelets (RPs).[1] RP counts are closely associated with thrombopoietic activities in human bone marrow and have been measured using flow cytometry[2-4]; however, their application in clinical settings has been hampered mainly due to technical issues.[5] Recently, RP is easily assessed in daily practice as immature platelet fraction (IPF) using an automated hematology analyzer. Clinical significance of IPF has been reported for differential diagnosis of thrombocytopenia,[6,7] evaluations of recovery from bone marrow-suppressive states (including hematopoietic stem cell transplantation) [8,9] and infectious complications, such as COVID-19.[10]

Thrombocytopenia is a common hematological abnormality in neonates, and its differential diagnoses are broad.[11,12] It is often difficult to identify the causal mechanism of thrombocytopenia in neonates due to the limited blood volume available for laboratory testing and the varied backgrounds of thrombocytopenia in the neonatal period. A bone marrow examination is the gold standard for evaluating megakaryopoietic activities,[13] but it is a rather invasive procedure for neonates. IPF may thus be a compelling alternative for the evaluation of thrombopoiesis,[14,15] although data on IPF in neonates are scarce.

The aim of this study was defined reference intervals (RIs) in neonates at birth, evaluated factors affecting IPF and analyzed the kinetics of IPF after birth to better understand IPF in neonates and determine its clinical usefulness.

Materials and Methods

Subjects

Neonates born between October 2018 and February 2020 in two tertiary hospitals in central Japan were consecutively recruited to this study. Neonates whose blood was drawn within 12 hours after birth and who received a complete blood count (CBC), including evaluation of IPF, were included. In some patients, samples on day14 and day28 after birth were also analyzed. Samples not suitable for measurement because of coagulation or any other reasons were excluded from the study.

This study protocol was approved by the institutional review board of Shinshu university and Nagano Children's hospital (approval number: 4409 and 31-19, respectively).

Measurements of IPF

The CBC, including the assessment of the percentage of IPF (IPF%), was performed with EDTA-2K-anti-coagulated whole blood samples using an automated hematology analyzer XN-1000 or XN-9000 (Sysmex, Kobe, Japan). The XN analyzer was equipped with a channel (PLT-F) specially designed to measure platelet parameters, and oxazine was used as a dye for nucleic acid staining in the system. The PLT-F consisted of a scattergram based on two-dimensional plots of forward side scatter (FSC) reflecting cell sizes and side fluorescence light (SFL) reflecting the amounts of RNA in platelets, which allowed the platelets and IPF to be more

specifically and accurately gated.[9,16] Immature platelets were differentiated from platelets counted with an algorithm based on the data derived from FSC and SFL, and IPF% was calculated as the ratio of immature platelets to total platelets. The absolute IPF value (A-IPF) was calculated as the total platelet number times IPF%.

Quality control procedures were daily performed following the manufacturer's instructions. The range of the coefficient of variance for the reproducibility of platelet counts and IPF% in each analyzer was 2.2%-2.7% and 3.5%-3.6%, respectively, during the study.

Statistical analyses

Comparisons between different groups were carried out using the Mann-Whitney U test, the Kruskal-Wallis test or Friedman's test, as appropriate. $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using the EZR software program.[17]

Results

Characteristics of the neonates

We analyzed a total of 822 blood samples from 533 neonates, including 423 on day 0, 203 at 14 days after birth and 196 at 28 days after birth (Supplemental Figure 1, Supplemental Digital Content 1). The platelet counts of 330 subjects assessed at birth were within the reference range,

while 46 neonates showed platelet counts below 150×10^9/L, and 47 showed platelet counts over 350×10^9/L. The median (range) number of platelets, IPF% and A-IPF in the 423 neonates were 256 (8-583) $\times10^9$/L, 3.2% (1.0%-26.3%) and 7.9 (2.1-57.1) $\times10^9$/L, respectively (Supplemental Figure 2, Supplemental Digital Content 2).

The demographics of the 330 neonates with normal platelet counts are shown in Table 1. The median (range) number of platelet, IPF% and A-IPF were 256 (151-350) $\times10^9$/L, 3.2% (1.0%-14.7%) and 7.9 (2.7-22.5) $\times10^9$/L, respectively (Supplemental Figure 3, Supplemental Digital Content 3).

Clinical factors and IPF%

Since the neonates in this study had different clinical backgrounds that might affect IPF%, we extract independent factors associated with IPF% to exclude patients with these factors for defining RIs of IPF. In the univariate analysis of 330 neonates with platelet counts within the RIs, the gestational age, birth weight, gender, neonatal asphyxia, intrauterine infections, chromosomal abnormalities and respiratory disorders were identified as candidate factors with $P < 0.15$ (Supplemental Table 1, Supplemental Digital Content 4). In the multivariate analysis, premature birth, neonatal asphyxia, intrauterine infections, chromosomal abnormalities, and respiratory disorders were identified as independent factors associated with IPF% (Supplemental Table 1, Supplemental Digital Content 4). Neonates with these factors showed

a higher IPF% than those without these factors (median: 3.3% vs. 2.8%, $P < 0.001$). We determined that 92 neonates without these factors affecting IPF% were suitable for RIs definition.

RIs of IPF

The RIs of IPF% and A-IPF were defined based on the 92 neonates without any clinical factors that might have been associated with IPF%, as identified above. The median (range) number of platelets, IPF% and A-IPF were 258 (151-350) $\times 10^9$/L, 2.8% (1.0%-6.7%) and 7.3 (2.7-20.4) $\times 10^9$/L, respectively. Since IPF% and A-IPF values were nonparametrically distributed, those values between the 2.5th and 97.5th percentiles were defined as the RIs for each: 1.3%-5.7% and 3.2-14.5 $\times 10^9$/L, respectively.

IPF changes after birth

In neonates without thrombocytopenia, the platelet counts at 14 and 28 days after birth were significantly increased compared to the counts on day 0 (Fig. 1A). IPF% and A-IPF peaked within the first two weeks and then decreased over the next two weeks while remaining higher than at birth (Fig. 1B and 1C).

Consecutive data of CBC and IPF at birth and 14 and 28 days after birth were available for the 26 subjects without factors affecting IPF%. Platelet counts increased until 14 days after

birth and then remained relatively unchanged until day 28 (Fig. 2A). IPF% and A-IPF both peaked on day 14 and then decreased until day 28 (Fig. 2B and 2C). On day 14, IPF% and A-IPF increased to about 2- and 3.3-fold the value at birth.

A comparison of IPF with reticulocytes after birth

To further understand the thrombopoietic changes after birth, we compared IPF with reticulocytes simultaneously measured after birth. Reticulocyte counts were available for 180 subjects at birth, 99 after 14 days and 102 after 28 days, all of whom had 150×10^9/L or more platelet counts. The hemoglobin value continuously decreased after birth, and the reticulocyte counts reached their lowest value at 14 days before increasing (Fig. 3).

In the seven subjects without any IPF-affecting factors and reticulocyte count data available, IPF% was highest and the reticulocyte count lowest on day 14 (Supplemental Figure 4, Supplemental Digital Content 5).

IPF in thrombocytopenic patients

A total of 19 patients were born with platelet counts $<100\times10^9$/L. The details of these cases are described in Supplemental Table 2, Supplemental Digital Content 6. In 16 patients, IPF% increased beyond the upper levels of the RIs, and IPF% was notably high in 2 cases of immune-mediated thrombocytopenia (case 17 and 19) with IPF% values of 18.7% and 26.3%,

respectively. However, other disorders were also associated with a high IPF% in some patients, suggesting that various conditions related to deliveries, neonatal backgrounds and immune status might affect thrombopoiesis and IPF in neonates.

Discussion

In this study, the RIs of IPF% and A-IPF among neonates were defined. IPF fluctuated after birth, significantly increasing until day 14 and then returning to almost the same level as at birth on day 28. Conversely, reticulocytes showed a different kinetics, significantly decreasing in the first two weeks after birth and then gradually increasing again.

We measured IPF with XN analyzers that have been widely utilized. Previous studies on IPF were mostly performed with an older version, the XE analyzer, from the same company as the XN analyzers. Although we did not directly compare these analyzers with regard to neonatal IPF measurements, IPF values measured with the XN analyzer have been shown to significantly differ from those obtained with the XE analyzer,[18] being more precise.[6,9] Therefore, care should be taken dealing with the analyzer utilized when evaluating IPF.

Using the XN analyzer, Ko et al.[18] reported RIs of IPF% between 1.9% and 7.3% in adults, and Sakuragi et al.[6] defined the upper limit of IPF% in healthy adults as 5.8%. The RIs of neonates based on our data were between 1.3% and 5.7%, suggesting no clinically meaningful difference in IPF RIs depending on the age, and IPF in neonates was able to be

evaluated using equivalent RIs of adults, although transient fluctuations in the first two weeks after birth need to be considered.

IPF is assumed to infrequently vary in healthy adult individuals.[19] However, IPF increased within the first two weeks after birth before returning to the baseline levels in the next two weeks in our study. Similar findings were previously reported,[20] and the chronological analyses of 26 subjects further confirmed this neonatal phenomenon within individuals. The thrombopoietin (TPO) surge after birth,[20-22] increased sensitivities of megakaryocytic progenitor cells for TPO[23,24] and other mechanisms might be related to this fluctuation.[25] A further understanding of the thrombopoietic systems with TPO-related and non-related signaling pathways in neonates is necessary. Furthermore, with comparison with reticulocytes, a transient increase was only observed in IPF but not in reticulocyte, suggesting that thrombopoiesis differ from erythropoiesis in neonates during the first month of life. Although continuous daily data was not obtained in this study, the schematic patterns of the assumed fluctuations of the two hematological parameters during the first month of life is shown in Fig. 4. Although thrombopoiesis and erythropoiesis are closely related, these hematopoietic lineage-specific differences would be intriguing when considered the hematopoiesis in the neonatal period.

Among 16 thrombocytopenic patients, 2 with antibody-associated, immune mechanisms, 1 with maternal ITP and 1 with anti-HLA alloimmune thrombocytopenia showed

an extremely high IPF%, which was consistent with previous reports,[26,27] although other conditions were also related to an increase in IPF%. Moreover, we identified several other factors potentially associated with higher IPF%, including premature birth, neonatal asphyxia, intrauterine infections, chromosomal abnormalities, and respiratory disorder, which needs further investigation.

Several limitations associated with the present study warrant mention. First, we recruited subjects from two tertial hospitals, and they might have differed from neonates without any complications, although we excluded subjects with potentially influential factors. Second, we only measured IPF using automated analyzers without other comparative methods, such as a flow cytometric analysis. Third, IPF measurements were not standardized. These issues merit further studies on IPF across multiple institutions with more subjects.

In conclusion, IPF in neonates can be evaluated essentially based on the same RIs as in adults, although some care should be taken when evaluating neonates in the first two weeks of life, due to the transient increase in IPF. IPF may be a useful tool for assessing thrombopoiesis and thrombocytopenia in neonates.

Author contributions

JK, MT and FI designed the study and wrote the paper. JK, YT, SS, NK, KS, YN and YH collected and analyzed the data. FI managed the study and analyzed the data. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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Figure legends

Figure 1. Platelet counts, percentage of the immature platelet fraction (IPF%) and the absolute IPF value (A-IPF) in neonates during the first four weeks after birth. Platelet counts (A), IPF% (B) and the A-IPF (C) were measured in 502 subjects without thrombocytopenia at birth and at days 14 and 28. Each box represents the median value with the 25^{th} and 75^{th} percentile range along with the 10^{th} and 90^{th} percentile values shown with dotted lines. Asterisks indicate *P* value with a significant difference.

Figure 2. A sequential analysis of platelet counts and IPF% and A-IPF in 26 neonates with normal platelet counts at birth. Platelet counts (A), IPF% (B) and A-IPF (C) of 26 patients with normal platelet counts at birth were sequentially measured on days 14 and 28. All of them were negative for clinical factors affecting IPF (see Results). Gray circles and dotted lines represent each case, and black circles and solid lines represent the median values. Asterisks indicate *P* value with a significant difference.

Figure 3. Hemoglobin value, reticulocyte percentage (RET%) and reticulocyte count (A-RET) in neonates after birth. Distributions of hemoglobin values (A), RET% (B) and A-RET (C) during the first four weeks after birth are shown. Each box represents the median value with the 25^{th} and 75^{th} percentile range along with the 10^{th} and 90^{th} percentile values shown with

dotted lines. Asterisks indicate *P* value with a significant difference.

Figure 4. Assumed hematological parameters fluctuations during the first month after birth. The platelet counts and IPF% peaked on day 14 and then decreased, while the hemoglobin value and RET% were highest at birth.

Supplemental Digital Content

Supplemental Digital Content 1. Supplemental Figure 1. Overall view of neonates and blood samples in this study. docx

Supplemental Digital Content 2. Supplemental Figure 2. Distribution of platelet counts, IPF% and A-IPF in 423 neonates. docx

Supplemental Digital Content 3. Supplemental Figure 3. Distribution of platelet counts, IPF% and A-IPF in 330 neonates with normal platelet counts. docx

Supplemental Digital Content 4. Supplemental Table 1. An analysis of clinical factors and IPF%. docx

Supplemental Digital Content 5. Supplemental Figure 4. A sequential analysis of the hemoglobin value, RET% and A-RET in seven cases without any IPF-affecting factors. docx

Supplemental Digital Content 6. Supplemental Table 2. Cases of thrombocytopenia (platelet counts $<100\times10^9$/L) at birth. docx

Figure 1

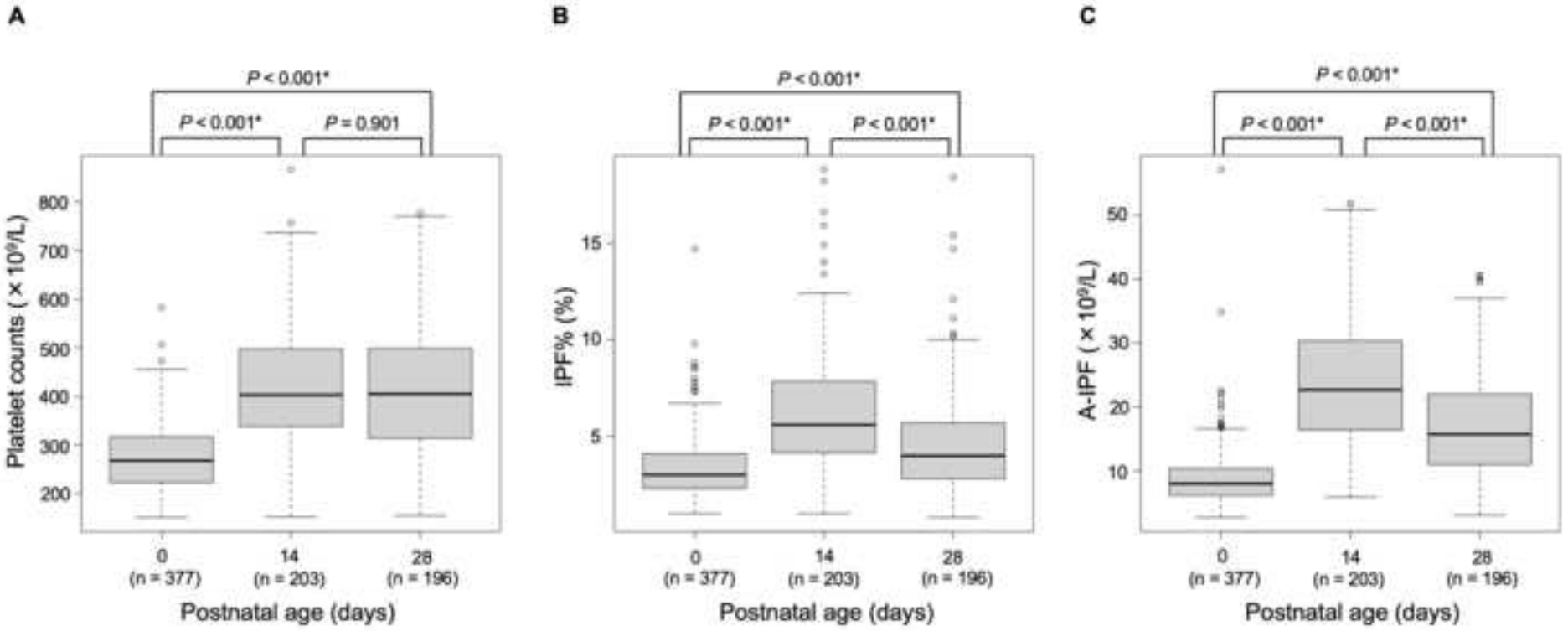

Figure2

Figure 2

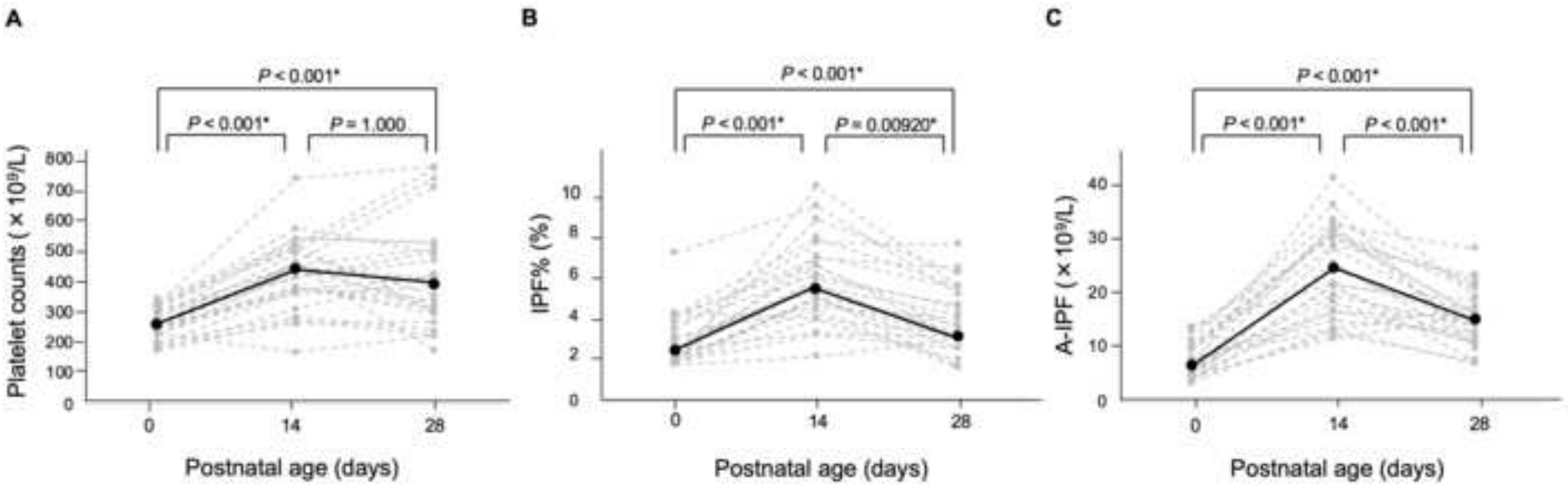

Figure 3

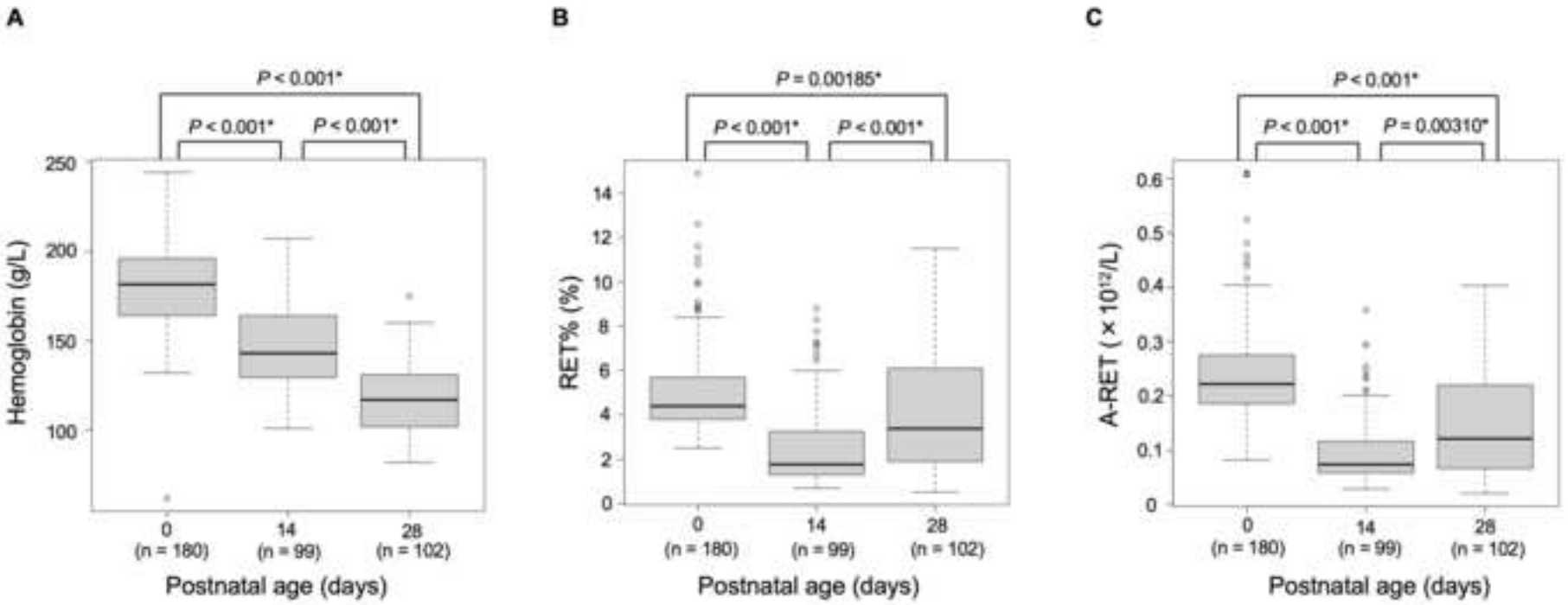

Figure 4

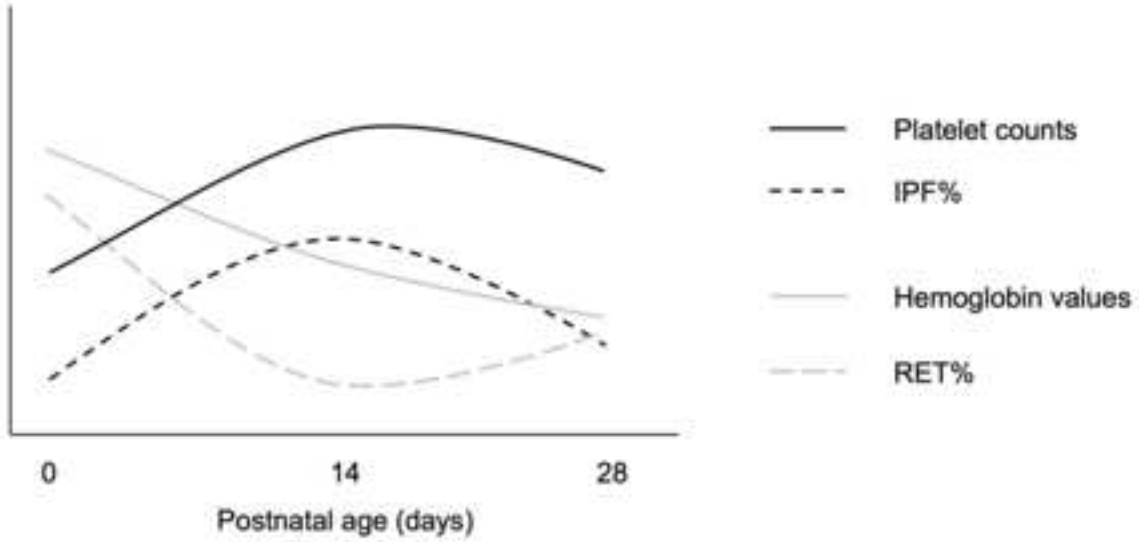

Table 1. Demographics of subject with normal platelet counts

	n=330
Male/female	183/147
WBC ($x10^9$/L); median (range)	13.15 (2.00-43.79)
RBC ($x10^{12}$/L); median (range)	4.79 (1.74-7.05)
Hemoglobin (g/L); median (range)	177(62-244)
Hematocrit (%); median (range)	51.2 (18.8-70.6)
Platelet counts ($x10^9$/L); median (range)	256 (151-350)
IPF% (%); median (range)	3.2 (1.0-14.7)
A-IPF ($x10^9$/L); median (range)	7.9 (2.7-22.5)
Complications	
Premature delivery (before 37 weeks)	135 (40.9%)
Low birth weight (< 2,500 g)	185 (56.1%)
Small for gestational age	67 (20.3%)
Premature rupture of the membranes	31 (9.4%)
Neonatal asphyxia	45 (13.6%)
Intrauterine infection	23 (7.0%)
Chromosomal abnormality	6 (1.8%)
Respiratory disorders	167 (50.6%)
Heart diseases	36 (10.9%)

IPF%; percentage of immature platelet fraction, A-IPF; absolute immature platelet fraction value