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Original Article

Establishment of reference interval for immature platelet fraction using Sysmex XN9000 hematology analyzer in Anhui province in China

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Abstract: The aim of this study was to establish reference intervals for immature platelet fraction (IPF) for healthy adults and term newborns in the area of Anhui province. Except outliers, a total of 2179 health adult examination subjects and 153 healthy term newborns were included in this study. The percentage (IPF%) and absolute value (IPF#) of IPF in adult peripheral blood and neonatal umbilical cord blood were measured using the Sysmex XN9000 fully automatic hematology analyzer. The reference intervals were calculated by non-parametric percentile method according to the Clinical and Laboratory Standard Institute guideline C28-A3. Our results showed that, for adults, no significant differences were found between males and females, while significant difference existed between different age groups for IPF% and IPF#. A significant difference also existed between adults and term newborns for all test parameters. The reference intervals of IPF% and IPF# for adults and term newborns were 0.7-8.4% and 2.9-16.9×10^9/L, 0.9-4.8% and 3.1-13.5×10^9/L, respectively. The reference intervals determined by the Sysmex XN9000 hematology analyzer for healthy adults and term newborns in the area of Anhui province would be useful for future clinical diagnosis as well as treatment.

Keywords: Reticulated platelet, reference interval, immature platelet fraction

Introduction

Reticulated platelet (RP) refers to the platelet that is newly released from the bone marrow, usually with higher coagulation activity [1]. RP can reflect the production of platelets by megakaryocytes in bone marrow. Therefore, it can help distinguish bone marrow failure from peripheral destruction in thrombocytopenia and can early predict bone marrow recovery after chemotherapy and transplantation; However, RP determination is lack of standardization of methodology [2]. With the progress of detection technology, researchers have found that there was a good correlation between immature plateletfraction (IPF) and RP in thrombocytopenia, and IPF can be measured simply and reliably on fully automated hematology analyzers, facilitating its clinical utility for diagnosing and monitoring thrombocytopenia [2, 3].

During the clinical practice, IPF is often measured by Sysmex hematology analyzers, such as XE-2100 and XE-5000 systems (Sysmex, Kobe, Japan). In those machines, the platelet counts are analyzed by impedance method and optical method, and IPF can be measured in the optical platelet channel using a fluorescentdye and a carefully designed gating system [4, 5]. The Sysmex XN9000 is a advanced fully automatic hematology analyzer using different principles, channels, and reagents from its previous versions and with better sensitivity and specificity for the measurement of IPF [6]. Considering its different principle for the measurement of IPF, we can assume that the previously established reference intervals for IPF may not be applicable for the SysmexXN. In this study, we wanted to establish new reference intervals for IPF on Sysmex XN9000. For this purpose, we measured IPF in the peripheral blood of 2179 health examination subjects and in the cord blood of 153 healthy term newborns using the XN9000 hematology analyzer in the area of Anhui province. These measurements

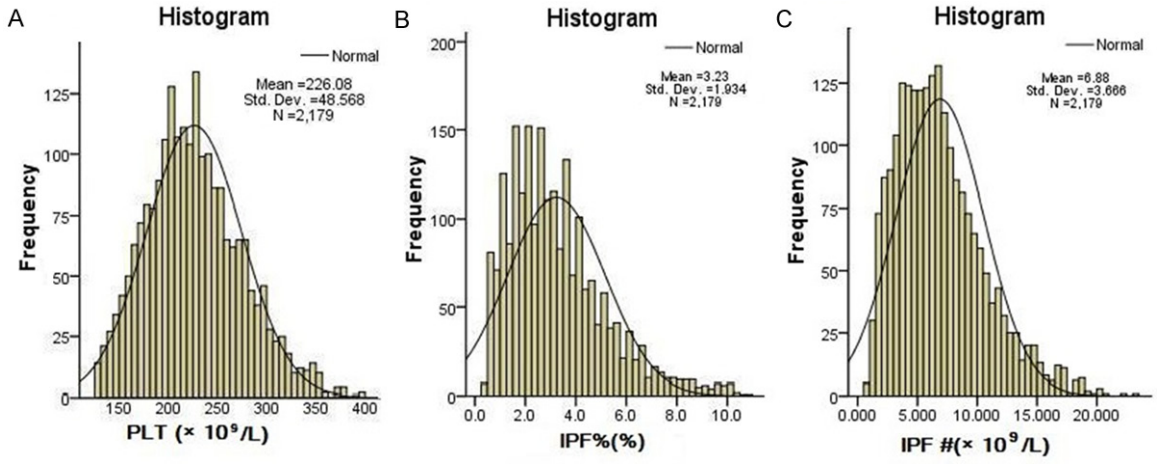

Figure 1. Histograms of PLT, IPF% and IPF# in healthy adults. The results demonstrated that the PLT count, the absolute values (IPF#) and percentages (IPF%) of IPF for the peripheral blood of healthy adults reflected a skewed distribution ($P<0.001$).

enable us to establish new normal references for IPF in this area and then play an important role in clinical diagnosis and treatment.

Materials and methods

Study participants

A total of 2202 health examination subjects who visited the First Affiliated Hospital of Anhui Medical University between March 2nd 2016 and April 3rd 2016 were included in this study. Participants met the following inclusion criteria were selected: a) in the physical examination, no significant abnormality was seen on internal examination, surgical examination, ECG, X-ray chest examination or B-ultrasonography examination, b) in the chemical test, individuals with white blood cell (WBC) $<10\times10^9$/L, Hb >120 g/L for male and >110 g/L for female, PLT $>125\times10^9$/L, without significant infectious symptoms or anemia. The exclusion criteria were: a) fasting blood level >6.11 mmol/L, TG level >1.7 mmol/L, cholesterol level >5.98 mmol/L, aspartate amino transferase level >40 U/L, alanine amino transferase level >50 u/L, creatinine >106 umol/L, urea >7.1 mmol/L, uric acid (female) >357 umol/L, uric acid (male) >428 umo/L, or the upper limit of urine red blood cells in female >10/HP.

Additionally, umbilical cord blood samples were collected from 158 healthy term newborns in our hospital between April 2015 and March 2016. Their median gestational week was 39.4 weeks (37.3-41.8 weeks), average birth weight was 3258 g (2502-4036 g). Their mothers were void of infectious disease, hereditary disease or hematologic disease, with a median age of 29 years (19-48 years).

This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the First Affiliated Hospital of Anhui Medical University. Written informed consent was obtained from all recipients or their legal guardians.

Collection and measurement of blood samples

EDTA-K2 anticoagulant peripheral blood samples and cord blood samples were respectively collected from health examination subjects and term newborns (2 ml each subject). Measurement was completed within a two hour timeframe after collection using the Sysmex XN9000 fully-automatic hematology analyzer (Sysmex, Kobe, Japan). Blood samples were analyzed according to the method previously reported to ensure that results of all parameters were available [7].

Statistical analysis

SPSS 19.0 software (SPSS Inc., IL, USA) was utilized to analyze all data. Data were expressed as median (min-max range). In addition to the exclusion criteria, outliers were further checked and eliminated as the method described previ-

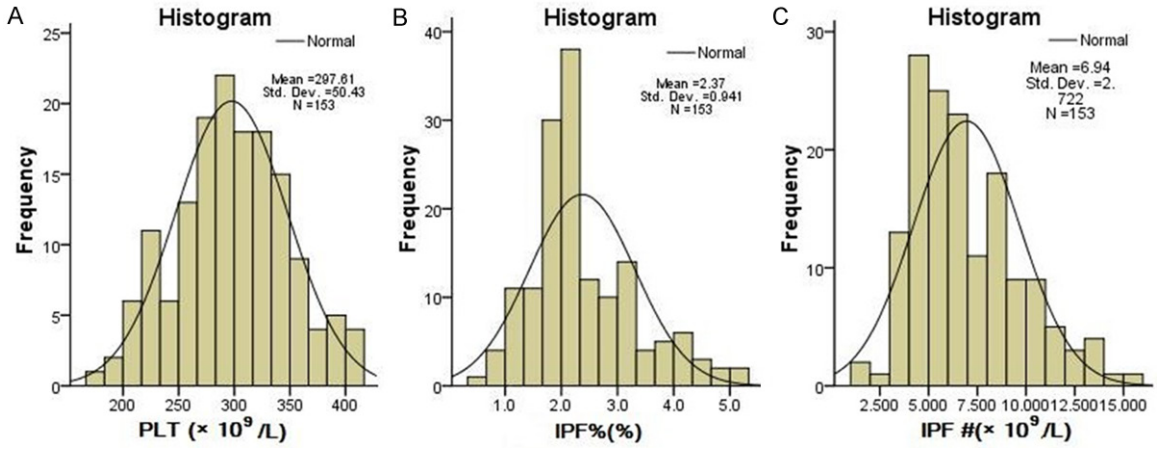

Figure 2. Histograms of PLT, IPF% and IPF# in healthy term newborns. The result demonstrated that the count of PLT, the absolute values (IPF#) and percentages (IPF%) of IPF for the cord blood reflected a skewed distribution (*P*<0.001).

Table 1. Parameters in different age groups in healthy adults

Group*	N (%)	PLT (×10⁹/L)	IPF% (%)	IPF# (×10⁹/L)
Lower age	1334 (61.2)	226 (127-396)	2.8 (0.4-10.4)	6.4 (0.8-23.0)
Middle age	655 (30.1)	218 (125-397)	3 (0.4-10.5)	6.6 (0.9-22.0)
Higher age	190 (8.7)	204 (125-373)	2.6 (0.5-10.8)	5.5 (1.1-19.0)
H value		42.394	7.773	13.852
P value		<0.001	0.021	0.010

*Note: lower age group: <40 years; middle age group: 40-60 years; higher age group: >60 years.

ously [8]. The distribution of platelet counts and IPF was examined for normality by the Kolmogorov-Smirnov Z-test. The reference intervals for IPF% and IPF# were calculated by a non-parametric percentile method (2.5th and 97.5th percentiles), according to the Clinical and Laboratory Standard Institute guideline C28-A3. Comparison of test parameters between male and female, adults and newborns, as well as different age groups, were performed using non-parametric Mann-Whitney U test or Kruskal-Wallis H test. *P* value less than 0.05 was considered statistically significant.

Results

Distribution analysis of immature platelet fraction in healthy adult and newborns

From a total of 2202 adult peripheral blood and 158 neonatal umbilical cord blood samples, 23 peripheral blood and 5 umbilical cord blood were considered as outliers and excluded. The remained 2179 adult peripheral blood and 153 umbilical cord blood samples were used to analyze. Histogram and Kolmogorov-Smirmov test for normality showed that PLT count, the absolute values (IPF#) and percentages (IPF%) of IPF for the peripheral blood of healthy adults and cord blood of term newborns reflected askewed distribution (P<0.001 for all). In healthy adults, the median values (min-max range) for platelet counts, IPF% and IPF# were 222×10⁹/L (125-397×10⁹/L), 2.9% (0.4%-10.8%) and 6.3×10⁹/L (0.8-23.0×10⁹/L), respectively (**Figure 1**). As for newborns, the median values (min-max range) for platelet counts, IPF% and IPF# were 292×10⁹/L (182-412×10⁹/L), 2.2% (0.6%-5.2%) and 6.4×10⁹/L (1.8-15.5×10⁹/L), respectively (**Figure 2**).

Distribution of immature platelet fraction in healthy adults at different age groups

The data reflected askewed distribution and expressed as median (min-max range). The comparison of test parameters between different age groups was performed by the non-parametric rank sum Kruskal-Wallis H test. We stratify the healthy adults into three groups according to age: the lower (<40 years old), middle(40-60 years old) and higher age group (>60 years old). In the lower age group, the median values for platelet counts, IPF% and IPF# were 226×10⁹/L (127-396×10⁹/L), 2.8%

Table 2. Parameters in adults (different genders) and term newborns

Variables	Healthy adults (n=2179)				Newborns (n=153)	*P* value*
	Total	Male (n=1138)	Female (n=1041)	*P* value		
PLT ($\times10^9$/L)	222 (125-397)	221 (125-373)	224 (125-397)	0.032	292 (182-412)	<0.001
IPF% (%)	2.9 (0.4-10.4)	2.9 (0.4-10.4)	2.8 (0.4-10)	0.232	2.2 (0.6-5.2)	<0.001
IPF# ($\times10^9$/L)	6.3 (0.8-23.0)	6.4 (0.8-23.0)	6.2 (0.8-22.0)	0.511	6.4 (1.8-15.5)	<0.001

Note: **P* value was calculated by comparing parameters between the healthy adults group and the newborns group.

Table 3. Reference intervals of test parameters in healthy adults and term newborns

Variables	Healthy adults (n=2179)				Newborns (n=153)
	Total	lower age (n=1334)	middle age (n=655)	higher age (n=190)	
PLT ($\times10^9$/L)	142-335	148-378	137-328	134-337	201-402
IPF% (%)	0.7-8.4	0.6-8.2	0.6-8.5	0.8-9.1	0.9-4.8
IPF# ($\times10^9$/L)	2.9-16.9	1.7-15.5	1.5-16.6	1.7-15.1	3.1-13.5

(0.4%-10.4%) and 6.4×10^9/L (0.8-23.0×10^9/L), respectively. In the middle age group, the median values for platelet counts, IPF% and IPF# were 218×10^9/L (125-397×10^9/L), 3.0% (0.4%-10.5%) and 6.6×10^9/L (0.9-22.0×10^9/L), respectively. In the higher age group,the median values for platelet counts, IPF% and IPF# were 204×10^9/L (127-396×10^9/L), 2.6% (0.5%-10.8%) and 5.5×10^9/L (1.1-19.0×10^9/L), respectively. The platelet count gradually decline with ageincreased. The middle age group had largest value of IPF% and IPF#, both decreased obviously in the higher age group (**Table 1**).

Distribution of immature platelet fraction in healthy adults and term newborn

The data between different genders reflected a skewed distribution, as a result the data were also expressed as median (min-max range). The comparisons between male and female, as well as adults and newborns, were performed using the non-parametric Mann-Whitney U test, as shown in **Table 2**. In the man group, the median values for platelet counts, IPF% and IPF# were 221×10^9/L (125-373×10^9/L), 2.9% (0.4%-10.4%) and 6.4×10^9/L (0.8-23.0×10^9/L), respectively. In the women group, the median values for platelet counts, IPF% and IPF# were 224×10^9/L (125-397×10^9/L), 2.8% (0.4%-10.0%) and 6.2×10^9/L (0.8-22.0×10^9/L), respectively. For platelet counts, there was a significant difference between men and women (P=0.032); as for IPF% and IPF#, no statistical difference was observed (P=0.232 for IPF%; P=0.511 for IPF#). Those result confirmed the PLT count of men and women should adopt different reference range, which was agreed with our daily work. According to our data, the distributions of IPF% and IPF# were not different between men and women, implying that sex-specific reference intervals are not necessary. During the comparison between adults and term newborns, we found that there was a significant difference existed regarding all three parameters (P<0.001 for all).

Reference interval for immature platelet fraction in healthy adults and term newborn

The reference intervals of IPF% and IPF# for healthy adults (for different age groups) and newborns were established by non-parametric percentile method according to the Clinical and Laboratory Standard Institute guideline C28-A3 (**Table 3**).

In the lower age group, the reference interval for platelet counts, IPF% and IPF# were 148-378×10^9/L, 0.7-8.4% and 2.9-16.9×10^9/L, respectively. In the middle age group, the reference interval for platelet counts, IPF% and IPF# were 137-328×10^9/L, 0.6-8.2% and 1.7-15.5×10^9/L, respectively. In the higher age group, the reference interval for platelet counts, IPF% and IPF# were 134-337×10^9/L, 0.6-8.5% and 1.5-16.6×10^9/L, respectively. In term newborns, the reference interval for platelet counts, IPF% and IPF# were 201-402×10^9/L, 0.9%-4.8% and 3.1-13.5×10^9/L, respectively.

Reference intervals comparison between our data and data from a Korean study

In a Korean study [9], authors used the same series Sysmex XN instruments to determine ref-

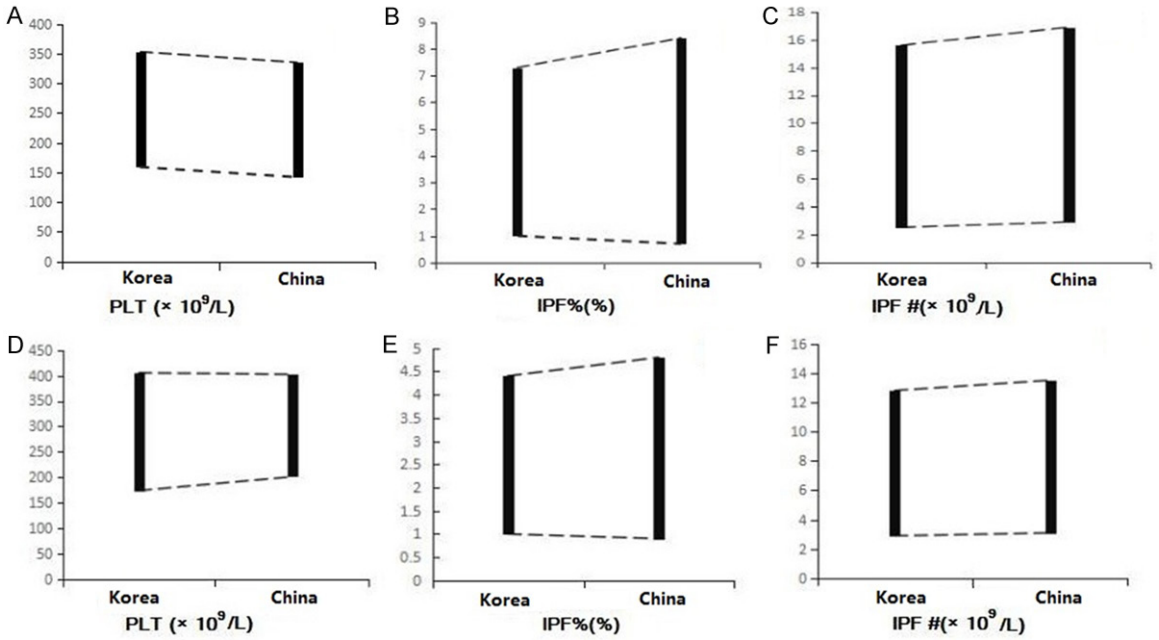

Figure 3. Comparison of reference intervals in our data with that of a Korean study using the same series of instrument (XN series Analyzer). A-C. Is for healthy adults. D-F. Is for cord blood. We compared the reference interval results of this study to a Korean study using the same series of instrument, and found that the test results roughly overlapped, but the range of results in our healthy adults was larger.

erence intervals of IPF for both healthy individuals and term newborns. The reference intervals established in that study for IPF% and IPF# on Sysmex XN were: 1.0-7.3% and 2.49-15.64×10^9/L in healthy individuals; and 1.0-4.4% and 2.94-12.82×10^9/L in term newborns. After comparison, we found IPF% and IPF# in healthy individuals had increased and widened reference intervals than that of the Korean; While, the reference intervals for the term newborns were almost same (**Figure 3**).

Discussion

Currently, in most Chinese clinical laboratories, the reference intervals of IPF were primarily determined by the manufacturers of reagents or instruments, or established based on the population in Europe, American or Japan, which indicated that the previously established reference intervals for IPF may not be applicable. Moreover, most of these earlier studies were restricted to mainly adults with very few about newborns. It is necessary, therefore, to establish the reference intervals suitable for specific region for both adults and newborns.

Studies have found that the hematological reference interval correlates significantly with age, gender, race, detection instrument or geographical factors [10], which suggests that factors such as age, gender, detection instrument and race should be seriously considered when establishing new reference intervals. In this study, we measured IPF in a large amount of healthy adults and healthy term newborns. The results showed that IPF%, IPF# and PLT count in healthy adults and cord blood of term newborns resulted in askewed distribution,with a reference interval in adults of 0.7-8.4%, 2.9-16.9×10^9/L and 142-335×10^9/L, respectively. There was no difference between man and woman groups except for PLT count (with *P* values of 0.232, 0.511 and 0.032, respectively); while, a significant difference existed between different age groups. We compared the reference interval results to that of other researchers and found that the test result ranges [9, 11, 12], for the same series of instruments, roughly overlapped (**Figure 3**), but the range of results in our healthy adults was larger. Detection instrument was another important factor influencing reference interval. For example, the IPF% and IPF# results for XE2100 was 0.5-3.3% and 1.25-7.02×10^9/L, respectively. The reasons for the difference might be as follows: a) As the areas, circumstances and population

are different, there exists a natural difference between reference intervals, b) Different instruments are based on different test principles. The XE2100 has only 2 PLT test channels: PLT-I and PLT-O, while the XN9000 has 3 channels to count PLT, specifically the PLT-F channel and the PLT-I and PLT-O channels. The PLT-F channel's fluorescent dye is oxazine, which could specifically dye DNA in the mitochondria of platelets, but it's dying extent for the non nucleic acid content, such as cell membrane, is much lower than PLT-O, consequently it has greater power to differentiate RBC fragments. Additionally, the power of the PLT-F channel to count PLT particles is five times greater than the PLT-I and PLT-O channels. And so, the reason that the PLT-F channel of XN9000 measures PLT with better precision and accuracy than PLT could be explained by either the test principles or the particles counted. Likewise, the measurement of IPF% and IPF# is also more specific and sensitive [13, 14]. The absolute value of reticulated platelet (IPF#) reflects the amount of immature platelets in the circulation, as a result it can reflect the timely production of platelets more specifically [15].

Neonatal thrombocytopenia is a common hematological disease in the neonatal period, with an incidence rate of 22%~35% in critical newborn [16]. Studies have shown that the most common reason for premature neonatal thrombocytopenia was chronic intrauterine hypoxia, often with intrauterine fetal growth retardation. The pathogenesis lies in the reduction in the megakaryocytes generation. Chronic intrauterine hypoxia produces a rise in the generation of erythropoietin, resulting in the hemocytoblasts over-differentiating into red blood cells while lower-differentiating into megakaryocytes [17-19]. Reticulated platelet can directly reflect the platelet-producing ability of the megakaryocytes, and it has a clear application prospect for the etiologic analysis of neonatal thrombocytopenia. Accordingly, it is also needed to establish reference intervals for IPF in healthy full-term neonates, and we further established the reference intervals for IPF% and IPF# in neonatal umbilical cord blood samples.

The reference intervals of IPF% and IPF# in cord blood have never been reported in China. In the current study, the amount of cord blood collected (153) was smaller relative to that of adults (2179). However, this number was sufficient for establishing a reference interval recommendation (≥120). According to our study, the reference interval of IPF% and IPF# for cord blood was 0.9-4.80% and 3.1-13.5×10^9/L, which was significantly differenced from the adult results ($P<0.001$). The results of cord blood were higher and wider than the adult results, which suggests that the platelet-producing ability of megakaryocytes in cord blood is more active.

Limitations for this study include: a) whether the cord blood could truly represent the results of newborns as the amount of cord blood samples was relatively little. We would confirm the results in future studies; b) we did not compare the results of PLT-O with PLT-F with instrumental methods, especially in patients with thrombocytopenia. It is accurate PLT and IPF measurement that could guide effective clinical diagnosis and treatment.

In conclusion, we established reference intervals of IPF% and IPF# for cord blood of healthy term newborns and peripheral blood of healthy adults in Anhui province for the first time in our country. Our results also suggested the establishment of reference intervals for different age groups was necessary. We hope this will provide a valuable reference frame for future clinical therapy and research.

Disclosure of conflict of interest

None.

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