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ORIGINAL ARTICLE

Immature platelet fraction measured on the Sysmex XN hemocytometer predicts thrombopoietic recovery after autologous stem cell transplantation

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Abstract

Objectives: A period of thrombocytopenia is common after stem cell transplantation (SCT). To prevent serious bleeding complications, prophylactic platelet transfusions are administered. Previous studies have shown that a rise in immature platelets precedes recovery of platelet count. Our aim was to define a cutoff value for immature platelets predicting thrombopoietic recovery within 2 d. *Methods:* Hematological parameters were measured on the Sysmex XN hemocytometer. We calculated reference change values (RCV) for platelets in eight healthy individuals as marker for platelet recovery. To define a cutoff value, we performed ROC analysis using data from 16 autologous SCT patients. *Results:* RCV for platelet concentration was 14.1%. Platelet recovery was observed 13 (median; range 9–31) days after SCT. Increase in immature platelet fraction (IPF) before platelet recovery was seen in all autologous SCT patients. Optimal cutoff IPF was found to be 5.3% for platelet recovery within 2 d (specificity 0.98, sensitivity 0.47, positive predictive value 0.93). *Conclusions:* We identified an optimal cutoff value for IPF 5.3% to predict platelet recovery after autologous SCT within 2 d. Implementing this cutoff value in transfusion strategy may reduce the number of prophylactic platelet transfusions.

Key words immature platelet fraction; stem cell transplantation; thrombopoiesis; platelets; transfusion

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The average platelet count in humans ranges between 150 and 350 $\times$ 10^9/L (1). Thrombocytopenia, that is, the state of decreased platelet concentration in peripheral blood, may be due to several causes, roughly divided into three main mechanisms: (i) aberrant distribution of platelets due to dilution, bleeding, or splenomegaly, (ii) increased platelet destruction or consumption, and (iii) a decreased platelet production. To correctly diagnose the cause of thrombocytopenia, it is crucial to distinguish between these three mechanisms (2). The assessment of thrombopoietic activity may be useful in differentiating between these three. Immature platelets, newly released from megakaryocytes in the bone marrow, are supposedly a marker of thrombopoietic activity: High immature platelet fraction (IPF) suggests increased thrombopoiesis, whereas low IPF suggests decreased thrombopoiesis (3, 4). Immature platelets decrease in size and RNA content as they age, analogous to reticulocytes (5, 6). Their characteristics allow us to differentiate immature from mature platelets, not only by flow cytometry, but also on the newest generation hemocytometers (3, 7–9). In 1969, Ingram and Coopersmith were the first to show an increase in immature platelets in mature beagles subjected to severe blood loss, reflecting increased thrombopoietic activity (5). Several more recent publications confirmed this observation (2, 10–12). This might also be of interest in patients undergoing stem cell transplantation (SCT) for hematological malignancies (7, 13–19). In the post-transplant period,

patients have a significantly lower concentration of platelets due to a decrease in thrombopoiesis. As platelets play an essential role in primary hemostasis, severe thrombocytopenia is associated with an increased risk of bleeding (20). Studies in thrombocytopenic patients have shown concordance between the onset of bleeding and platelet count. A minimum platelet count of 5×10^9/L is thought to be sufficient to maintain vascular integrity and prevent severe bleeding (21, 22). Prophylactic platelet transfusions are administered to prevent severe and life-threatening bleeding in thrombocytopenic patients (23). According to current consensus, prophylactic platelet transfusions are ordered when platelet count falls below a threshold of 10×10^9/L (23–25). Disadvantages of prophylactic transfusions may be major and minor adverse effects for patients, varying from mild transfusion reaction to anaphylaxis or severe infection. Also, high costs and the future shortage of donors should be kept in mind (23). Furthermore, it is debatable whether this prophylactic strategy is actually necessary. Some studies suggest that a therapeutic transfusion strategy is safe in a selected group of stable patients without sepsis, infections or an increased bleeding risk (23, 26). A previous study even suggested that platelet transfusions may inhibit thrombopoiesis by binding thrombopoietin (27). A strategy to reduce platelet transfusions in thrombocytopenic patients after SCT may be based on a better prediction of the thrombopoietic recovery. If a reliable prediction of the natural recovery of the platelet number within a few days is feasible, a valid decision whether or not to give a prophylactic platelet transfusion would be facilitated. The number of immature platelets could be used as such a predictor, as various studies have described a rise in the number of immature platelets shortly preceding platelet recovery (13–17, 28). Some studies also proposed a cutoff value for immature platelets predicting platelet recovery (18, 29). Different definitions of platelet recovery were used in combination with a less standardized, more time-consuming method of measurement of immature platelets. A fundamental step toward the implementation of immature platelets in transfusion management after SCT is defining a reliable cutoff value based on a standardized technique. The aim of this study was to define a cutoff value for immature platelets predicting thrombopoietic recovery using the novel Sysmex XN analyzer (Sysmex corporation, Kobe, Japan).

Methods

Patients

For a period of 8 months (September 2012 through April 2013), eighteen adult hemato-oncological patients, scheduled for autologous SCT in the Maastricht University Medical Center, were included. The number of venous blood draws and transfusion regime were not influenced by our observational study. Our study was approved by the local ethics committee. Patients could object to the use of their medical information for research purposes. Two patients were excluded due to missing data (i.e., day of platelet recovery not recorded). Sixteen patients were included in the final data analysis. During hospital stay, bleedings were reported and classified according to the WHO bleeding scale (30).

Sample collection

Venous whole-blood samples were collected in K2-EDTA Vacutainer tubes (BD Diagnostics, Plymouth, UK). Hemato-oncological patients admitted to the ward were routinely sampled daily around 8 AM. When samples were drawn more frequently, only the 8 AM samples were used for our analysis. Samples were stored at 4°C for a maximum of 51 h until analysis. A previous study showed that the IPF in samples with K2-EDTA stored at 4°C stays within precision limits over 72 h (31). We confirmed this finding by an additional experiment. Patient data were collected between start of conditioning regimen and day of platelet recovery.

Measurement of immature platelets

Whole-blood, EDTA anticoagulated, samples were measured on both the Sysmex XE-5000 analyzer (Sysmex corporation, Kobe, Japan) and the Sysmex XN analyzer. Earlier Sysmex hematology analyzers (such as the XE-5000) use the RET-channel for the measurement of immature platelets and the optic platelet count (PLT-O). Impedance (PLT-I) is an alternative method for measuring platelets. The Sysmex XN has, in addition, a novel PLT-F-channel for measurement of both mature and immature platelets. This PLT-F-channel was introduced to specifically gate platelets for a more accurate platelet count (PLT) and IPF. On both analyzers, an algorithm decides which platelet count was reported. Measurement of immature platelets is similar on both analyzers and is based on the principle of hemocytometry. After perforation of platelet cellular membranes by reagents, nucleic acids are stained by fluorescent dyes: polymethine and oxazine (XE-5000) or oxadine (XN; 31, 32). An algorithm defines the gating of mature and immature platelets. This algorithm contains side fluorescence (reflection of RNA content), side scattered light (information on intracellular structure), and forward scattered light (information on cell size).

Platelet transfusions

Platelet transfusions were given in a standardized fashion according to the national guidelines. Prophylactic transfusion trigger was defined by a platelet concentration of $<10 \times 10^9$/L. In case of clinical complications, surgical intervention or the use of anticoagulants the transfusion trigger may be higher (33). Patients in the SCT protocol received irradiated platelet products, prepared from the buffy

coats of five different ABO/D-matched whole-blood donors, resuspended in one unit of plasma to a volume of circa 350 mL and containing at least 250×10^9 platelets (33, 34). None of the patients received HLA compatible apheresis platelet concentrates. Some patients received pathogen-inactivated platelets (treated with riboflavin/UVB-pathogen) (Mirasol; TerumoBCT, Lakewood, CO, USA; see Table 2). Transfusion reactions were reported according to the standard protocol.

Biological variation, RCV, and platelet recovery

To assess biological within-person variation, PLT, IPF, and the absolute number of immature platelets (IPA) were measured in eight healthy subjects (four men and four women, age 23–33) once a day at the same time on 5 d in duplicate on the Sysmex XN and XE analyzers. Twofold nested ANOVA was used to estimate, between-person (CV_{bp}), biological (within-person) (CV_{wp}), and analytical (CV_a) coefficients of variation including 95% confidence intervals (35). RCV was calculated based on the biological and analytical coefficients of variations found in this experiment. RCV is calculated using the formula—$RCV = Z \cdot \sqrt{\left[2 \cdot \left(CV_{wp^2} + CV_{a^2}\right)\right]}$ —and reflects the minimal difference required to define a statistically significant increase or decrease compared with the previous result in that person. Z is the number of standard deviations appropriate to the desired probability (36, 37). In this study, we used a Z-score of 1.96 for $P < 0.05$. Platelet recovery was defined as the first day that platelet count increased without transfusion and exceeding the RCV.

Statistical analyses

Statistical analyses were performed with SPSS Statistics version 20 (IBM corporation, New York, NY, USA). Sensitivity, specificity, and positive predictive value (PPV) were calculated as described by Emir *et al.* (38), where sensitivity, specificity, and PPV for our patient population are weighted averages. Weights we used are based on the number of patient samples as a control (no recovery within 2 d) or as a case (recovery within 2 d). We analyzed sensitivity and specificity of both IPA and IPF from day 8 after SCT until the day of platelet recovery. Using these data, we constructed receiver operating characteristics (ROC) curves.

Results

Biological variation

Figure 1 shows the mean concentrations and absolute ranges for PLT, IPA, and the IPF of eight healthy individuals measured as described in the methods section. Using these data, we calculated analytical, within-person, and between-person coefficients of variation for PLT, IPA, and IPF. Results are shown in Table 1.

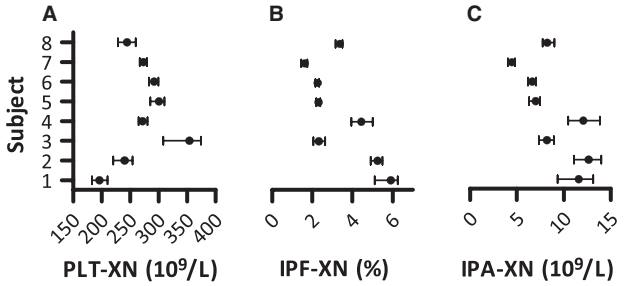

Figure 1 Biological variation. Mean concentrations (dots) and absolute range (horizontal lines) of platelet count (PLT) (panel A), the immature platelet fraction (IPF) (panel B) and the absolute number of immature platelets (IPA) (panel C) measured in healthy individuals ($n = 8$) on 5 d in duplicate. Subjects are numbered in a random order.

Table 1 Coefficients of variation and RCV

	PLT	IPF	IPA
$RCV_{95\%}$	14.12	23.44	33.63
CV_a	0.58 (0.48–0.74)	4.04 (3.31–5.16)	4.20 (3.45–5.37)
CV_{wp}	5.06 (4.06–6.70)	7.43 (5.69–10.14)	10.60 (8.33–14.27)
CV_{bp}	17.21 (11.25–35.26)	45.86 (30.20–93.55)	32.91 (21.43–67.53)

Reference change values (RCV), analytical coefficient of variation (CV_a, 95% confidence interval), within-person coefficient of variation (CV_{wp}, 95% confidence interval), and between-person coefficient of variation (CV_{bp}, 95% confidence interval) for platelet count (PLT), immature platelet fraction (IPF), and the absolute number of immature platelets (IPA) based on our biological variation study.

Patient characteristics

Sixteen patients who underwent autologous SCT were included in the study. All of them received a peripheral blood SCT. One suffered WHO grade 2 bleeding, a combination of epistaxis and petechiae. One transfusion reaction was reported: a patient experienced chills after platelet transfusion. No infectious cause was identified in this case. Patient characteristics are shown in Table 2.

Platelet recovery

Platelet recovery (an increase in platelets greater than its RCV and not due to platelet transfusion) was observed after a median of 13 d in patients after autologous SCT. Before platelet recovery, we observed an increase in IPA exceeding RCV, that is, more than 33.63%, in all patients. This increase in IPA measured on the XN analyzer preceded platelet recovery by a median of 3 d (range 1–6 d). Increase in IPF exceeding RCV, that is, more than 23.44%, was also observed in all patients prior to platelet recovery. This increase in IPF measured on the XN analyzer was seen by a

Table 2 Patient characteristics

	Autologous PBSCT (n = 16)
Male gender (%)	63
Age (yrs)	59 (44–66)
Diagnosis	AL-amyloidosis (n = 2)
	AITL (n = 1)
	Follicular lymphoma (n = 2)
	Hodgkin lymphoma (n = 1)
	MM (n = 7)
	DLBCL (n = 3)
Conditioning regimen	BEAM (n = 6)
	HDM (n = 9)
	Cyclophosphamide, Busulfan (n = 1)
Days till platelet recovery	13 (10–16)
Number of platelet transfusions per patient	1 (1–5)
Pathogen-inactivated platelet transfusions	2
Bleeding complications	WHO grade 2 (n = 1)

Median and range.
AITL, angioimmunoblastic T-cell lymphoma; BEAM, BCNU, cytarabine, etoposide and melphalan; DLBCL, diffuse large B-cell lymphoma; HDM, high-dose melphalan; MM, multiple myeloma; PBSCT, peripheral blood stem cell transplantation.

median of 4 d (range 2–7 d) before platelet recovery. Figure 2 shows the course of PLT, IPA, and IPF in a patient after autologous SCT.

ROC analysis

ROC curves were constructed for all patients. IPA and IPF, as well as XN and XE data, were analyzed separately. ROC curve for IPF from day 8 till the moment of platelet recovery after autologous SCT measured with the XN analyzer showed an area under curve (AUC) of 0.86. ROC curves for the IPA, based on data from the Sysmex XE-5000, had lower AUCs. ROC curves are shown in Fig. 3. AUCs are shown in Table 3.

Our aim was to define a cutoff value with high PPV. ROC curve for IPF after autologous SCT measured on the Sysmex XN showed the most optimal AUC. We determined a cutoff value of 5.3% [sensitivity 0.47 (95% CI 0.29–0.65), specificity 0.98 (95% CI 0.85–1.10), and PPV 0.93 (95% CI 0.81–0.1.06)] to predict platelet recovery within 2 d measured on the Sysmex XN.

Table 3 AUCs of ROC curves based on estimated sensitivity and specificity for the IPF and the absolute number of immature platelets (IPA) measured on both the Symex XE-5000 and the XN analyzer

	AUC
IPA-XN	0.83
IPA-XE 5000	0.71
IPF-XN	0.86
IPF-XE-5000	0.66

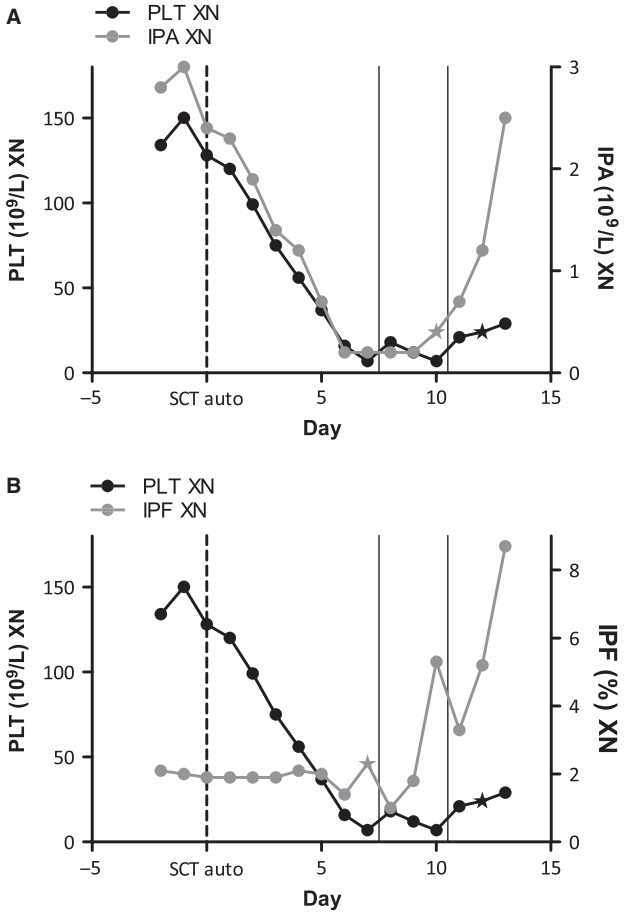

Figure 2 Representative example of the course of platelet count (PLT), the absolute number of immature platelets (IPA), and immature platelet fraction (IPF) in a patient from stem cell transplantation (SCT) until recovery. Panel A shows the course of the PLT (black) and the IPA (gray). Panel B shows the course of PLT (black) and the IPF (gray). Dots represent concentrations in morning samples. Dotted vertical line indicates day of SCT. Continuous vertical lines indicate platelet transfusions. Stars indicate an increase (in PLT, IPA, or IPF) exceeding its reference change value (RCV) not due to platelet transfusion.

Discussion

In patients awaiting thrombopoietic recovery after SCT, a strategy of prophylactic platelet transfusion is considered best practice, despite questions and debates about dose and lowest acceptable platelet counts (21, 22, 39). Platelet transfusions, although generally accepted as safe, may cause various transfusion reactions and are considered a scarce and also costly resource (23). In search of sensitive predictors of thrombopoietic recovery, the feasibility of immature platelets measured by the Sysmex XN hematocytometer was studied as a tool to detect early platelet recruitment.

An important step toward determination of a cutoff value for immature platelets is to objectively define platelet recovery. We used a validated statistical approach based on

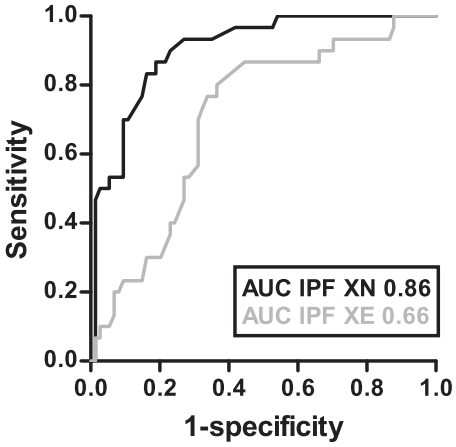

Figure 3 ROC curves and AUCs of immature platelet fraction (IPF) measured on the Sysmex XE-5000 and XN analyzer after autologous stem cell transplantation (SCT).

biological and analytical variation to objectify platelet recovery based on RCV (36, 37). A possible limitation may be that we assumed biological within-subject variation to be the same for healthy subjects as for patients after SCT. However, this assumption appears valid because a previous study showed that for the majority of parameters, biological within-subject variation is of the same order in health and disease (40). This is likely not true for analytical variation. Nevertheless, Fraser (41) showed that a ratio of 0.50 of analytical variation to within-subject variation has an effect of only 12% on RCV. While within-subject variation is much larger than analytical variation in our study, differences in analytical variation will have a negligible effect on RCV.

Previous studies used different definitions for platelet recovery, for example, platelet count $\geq 30 \times 10^9$/L for three consecutive days, seven consecutive days with a platelet count $\geq 20 \times 10^9$/L without platelet transfusions, or a spontaneous increase in PLT count without platelet transfusions (13–15, 18, 28). Despite the fact that we used RCV to define platelet recovery, the interval between increase in immature platelets and platelet recovery we found appears similar, that is, approximately 2 d (15, 16, 18, 28).

To estimate sensitivity, specificity, and PPV, we used the approach proposed by Emir *et al.* (38) to use multiple time points for each patient. We included all morning samples from day 8 after SCT until platelet recovery. This may be a limitation, as it led to a different number of controls per patient: more controls when recovery time is longer, which in turn led to higher specificity. Day 8 was chosen because we observed earliest platelet recovery 2 d later (day 10).

ROC curves based on estimated sensitivity and specificity showed differences in AUCs between IPF and IPA and between measurements on the Sysmex XE-5000 and the Sysmex XN. ROC curve of IPF measured on the Sysmex XN had a larger AUC compared to XE-5000. This reflects that IPF measured by the XN has a better precision than when measured by the XE-5000.

We estimated a cutoff value of IPF 5.3% in patients after autologous SCT to predict thrombopoietic recovery within 2 d, with a PPV of 0.93. Based on the large between-subject variations of 32.9% and 45.9% for IPA and IPF, respectively, found in our biological variation study, it would be reasonable to define a cutoff value indicating the difference between two measurements (or delta). However, ROC curves based on both absolute and delta values for IPA and IPF did not have acceptable AUCs, and this method was therefore not further investigated (data not shown).

In only one other study, a cutoff IPF predicting platelet recovery after autologous SCT was estimated (18). In this study, containing 31 patients, an IPF greater than 7.0% on day 8 after SCT predicted platelet recovery within 4 d (PPV 79%, sensitivity 76%). Because immature platelets were assessed using flow cytometry, it is difficult to compare this result with our findings.

The cutoff value for IPF of 5.3%, with high PPV and specificity, is based on observational data. Theoretically, this cutoff may be used to decide whether or not to give a platelet transfusion. Platelet transfusion may be omitted when platelet count is likely to recover within 2 d. This strategy may reduce the number of platelet transfusions and its associated risks and costs.

The hemostatic capacity of immature platelets is a point of interest, because current prophylactic strategy is intended to prevent serious bleeding incidents. Previous studies suggest that thrombocytopenic patients with high concentrations of immature platelets of up to 40–50%, such as ITP patients, have a smaller bleeding-tendency compared with thrombocytopenic patients with low concentrations of immature platelets, such as chemotherapy-treated patients (42). This is suggestive of a higher hemostatic capacity of immature platelets. Clearly, clinical interventional studies are needed to address this issue proving more evidence for the value of high IPF in platelet transfusions strategies.

Another argument to include a marker of thrombopoiesis in transfusion strategy is the observation that thrombopoiesis may be suppressed by transfused platelets (7, 15, 27, 43). However, while previous studies reported a decrease in IPF after platelet transfusion (7, 15), we did not observe an unequivocal effect of platelet transfusions in our population. A recent study by Bat *et al.* (44) showed that platelet transfusions lead to a decrease in IPF, but do not alter the IPA, suggesting dilution by an increase in circulating mature platelets. Our finding was that immature platelets are sometimes increased and sometimes decreased after platelet transfusion. The dilution effect of platelet transfusion on immature platelets (e.g., increase or decrease) depends on both the immature platelet count before transfusion and the immature platelet content of the platelet concentrate (17, 45).

In summary, we demonstrated an increase in immature platelets measured on the Sysmex XN analyzer preceding platelet recovery. We showed that IPF cutoff of 5.3% is a promising predictor of platelet recovery in patients after autologous SCT. However, a small number of patients were included in the final data analysis. Due to the observational design of the study, the relationship between immature platelets and bleeding risks could not be evaluated. Interventional clinical trials in a multicenter setting are required to validate the cutoff value presented in this study and to establish its role in IPF-based transfusion policies.

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