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ORIGINAL ARTICLE

Can Immature Granulocyte Counting be Utilized in the Selection of Samples for Flow Cytometric Enumeration of Stem Cells?

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SUMMARY

Background: **This study aimed to compare the IMG counting by an auto hematology analyzer with the flow cytometric enumeration of CD34+ cells.**
Methods: **All data from 124 samples submitted to the hematology laboratory for CD34+ cell counting in 2019 and 2020 were retrospectively evaluated. Whole blood samples were taken into EDTA tubes and assayed within 2 hours. The numbers and percentages of WBC and IMG were determined by using Mindray BC6200 hematology analyzer, while the same were determined for CD45 and CD34+ cells by using Beckman Coulter Navios flow cytometer. The performance of the new method was determined by the receiver operating characteristic (ROC) analysis.**
Results: **Out of the 124 samples, 94 were collected from healthy individuals and 30 were collected from patients. A significant correlation was found between IMG and CD34+ cell counts in all patients (r = 0.71, p = 0.000) at the cutoff values of > 20/µL and > 50/µL. The Bland-Altman analysis of all patients showed that there was an agreement between the two methods. When the cutoff value of 20/µL for CD34+ cells was used in the ROC analysis, the sensitivity and specificity were calculated as 100 (96.1 - 100) and 96.77 (83.3 - 99.9), respectively, for the IMG count of > 900/µL.**
Conclusions: **An IMG count of 900/µL can be used as the cutoff value in the analysis with the Mindray BC6200. The IMG counting cannot replace the flow cytometric CD34+ cell enumeration but can be used in the selection of the samples for stem cell enumeration by flow cytometry.**
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KEYWORDS

CD34, stem cell, immature granulocyte, flow cytometry, hematology analyzer

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an important treatment method for various diseases, in particular hematological neoplasms. Hematopoietic stem cells can be obtained from bone marrow, peripheral blood, or cord blood [1]. One of the most important factors affecting HSCT outcomes is the number of stem cells infused [2]. The number of stem cells to be infused

is determined by the flow cytometric enumeration of live CD34+ stem cells [3,4].
Peripheral blood stem cell collection by apheresis is a less invasive approach compared to other methods and is a frequently used method in HSCT. As the collection of stem cells requires adequate mobilization from the bone marrow compartment, tests to assess the mobilization are commonly used. Although the flow cytometric enumeration of CD34+ stem cells in peripheral blood is a validated approach for stem cell counting, it has some disadvantages, including high cost, technical difficulties, and long run times. [5]. In particular, samples without a sufficient number of CD34+ cells in the peripheral blood make the use of flow cytometry for stem cell counting ineffective. To avoid ineffectual CD34+ stem cell counting, there is a need for faster and inexpensive methods that can be used for the selection of samples before flow cytometry. Automated hematology analyzers are readily available, inexpensive, easy to use, and deliver analysis results within minutes after sample collection. Automated hematology analyzers use the following techniques: forward laser scatter, side laser scatter, and fluorescence. Technological advances allowed automated hematology analyzers not only to perform WBC differential but also to identify and quantify the immature granulocytes, using parameters such as immature granulocytes (IMG), high-fluorescent cells (HFC), and stem cell index (SCI). Studies available in the literature show that there is an agreement between the hematopoietic progenitor cell (HPC) and SCI parameters by different automated hematology analyzers and the flow cytometric CD34+ stem cell enumeration [6-9]. However, the correlation values range from 0.45 to 0.81 among the hematology analyzers included in the comparison [10,11]. The Mindray BC 6200 is an automated hematology analyzer that can perform IMG counting. IMG parameter is a fast and inexpensive method and has the potential to estimate the number of CD34+ stem cells in peripheral blood samples. To date, no studies performed on this matter are available in the literature.
The present study aimed to investigate the correlation between the IMG counting by Mindray BC 6200 auto hematology analyzer and the flow cytometric CD34+ stem cell enumeration, and thus the use of IMG counting in the selection of the samples for stem cell enumeration by flow cytometry.

MATERIALS AND METHODS

Study design

This study was conducted upon obtaining (protocol no. 2021-01/938) the Ethics Committee approval from Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital.
In the study, the demographic and laboratory data of 124 samples submitted to the hematology laboratory for stem cell counting in 2019 and 2020 were retrospectively evaluated. The study included 94 samples from healthy individuals and 30 samples from patients. Whole blood samples were taken into EDTA tubes and hemogram, and flow cytometry analyses were performed within 2 hours in the respective order. The numbers and percentages of WBC and IMG were determined by using Mindray BC 6200 hematology analyzer (Mindray, Shenzhen, China). The number and percentages of CD45 and CD34 cells were determined by using a Beckman Coulter Navios flow cytometer (Brea, CA, USA).

Flow cytometric analysis

The tube for the CD34+ stem cell counting was labeled with the patient's name and the antibodies contained in it. The antibodies (CD34 PE, CD45 FITC, 7-ADD PC5, Beckman Coulter) were pipetted into the labeled tubes following the leaflet, and a patient sample of 100 µL was added to them. The tubes were incubated for 15 minutes in the dark at room temperature. At the end of the incubation period, the tubes were vortexed and 2 mL Lysing Solution (Beckman Coulter) was added. After vortexing the samples, the tubes were incubated for the second time, for 10 minutes in the dark at room temperature. Then, 100 µL Stem-count fluorospheres (Beckman Coulter) were added and the tubes were vortexed and read on a 10-color Beckman Coulter Navios flow cytometer. 100,000 events were collected, and CD34+ stem cell percentages and numbers were determined by using the Kaluza software (Treestar, Ashland, OR, USA). The measurement and analysis procedures were performed according to current guidelines [12]. The device performance was checked by daily flow checks (Beckman Coulter). The intra-day and inter-day coefficient of variation (CV) was < 3%. The accuracy of the system was monitored monthly by using the UK NEQAS external quality assessment program.

Complete blood count

In the complete blood count using the "SF Cube" technology, cell size was measured with "Forward Laser Scatter", cell structure was determined with "Side Laser Scatter", and the DNA/RNA density in the cells was measured with "Fluorescence signals". Three-dimensional cell analyses were performed in the DIFF module of the five-channel BC 6200 system. The M-6 LD Lyse (Mindray) and M-6 FD Dye (Mindray) reagents were used. The total WBC count was measured by counting all the particles in the WNB channel in the WBC region, and then the number and percentages of immature granulocytes (IMG# and IMG%, respectively) were determined in the DIFF channel. The performance of the Mindray BC 6200 analyzer was monitored by daily internal quality assessments. The intra-day and inter-day CVs were 0.9 % and 1.2 %, respectively. The accuracy of the system was monitored monthly by using the AplusTBD external quality assessment program.

Table 1. Demographic data of patients and healthy donors.

Demographic data	n	Gender, n F/M	Age (years) median (min - max)
All individuals	124	41/83	34 (18 - 63)
Healthy donors	94	32/62	32 (18 - 53)
Patients	30	9/21	50 (24 - 63) *
Multiple myeloma	13 (43%)		
Non-Hodgkin lymphoma	8 (27%)		
Hodgkin lymphoma	6 (20%)		
Acute lymphoblastic leukemia	2 (7%)		
Solid tumor	1 (3%)		

*** There is a significant difference in age between patients and healthy donors ($p < 0.001$).**

Table 2. Correlation coefficients between the IMG count and the CD34 count at different cutoff values.

	IMG #	
CD34 #	Pearson Correlation (r)	p-value
All subjects	0.71	0.000
< 20/µL	0.27	0.141
> 20/µL	0.549	0.000
< 50/µL	0.657	0.000
> 50/µL	0.433	0.000

Statistical analyses

The IBM SPSS, version 23, software and the MedCalc (MedCalc Software, Mariakerke, Belgium) trial version were used. Numerical variables were presented as mean and standard deviation and categorical variables as frequency and percentage. The normality of the data was tested by the Shapiro-Wilks test. The Pearson correlation efficient or Spearman correlation efficient was used to test the linear relationship between the two numerical variables. The agreement between the methods was evaluated by using the Bland-Altman plot. The performance of the new method was determined by the receiver operating characteristic (ROC) analysis. The optimum cutoff values were calculated by summing up the maximum sensitivity and specificity values. A p-value of < 0.05 was considered statistically significant.

RESULTS

Out of the 124 samples, 94 were collected from healthy individuals and 30 were collected from patients. While the gender distribution was not significantly different between the groups, the mean age of the patient group was significantly higher than of the group of healthy individuals. The most common diagnosis observed in the patient group was multiple myeloma (n = 13, 43%). Demographic data are presented in Table 1.

The FS/SS/FL dot plot showing the WBC differential and the measurement of IMG parameter by automated hematology analyzer and the SS/CD34 dot plot of the flow cytometric CD34+ stem cell enumeration are shown in Figure 1 and 2, respectively.

There was a statistically significant difference between the mean of IMG numbers and the mean of CD34+ stem cell numbers ($p = 0.000$). In addition, a significant correlation was found between the IMG numbers and the CD34+ stem cell numbers in all patients ($r = 0.71$, $p = 0.000$). A significant correlation was found between the IMG numbers and the CD34+ stem cell numbers at cutoff values of > 20/µL and > 50/µL, as shown in Table 2.

According to the Bland Altman analysis performed for all patients, an agreement was observed between the two methods, as shown in Figure 3.

In the ROC analysis, when a cutoff value of 20/µL was taken for the number of CD34+ cells, the sensitivity was 100 (96.1 - 100) and the specificity was 96.77 (83.3 - 99.9) for the IMG count of > 900/µL (Figure 4).

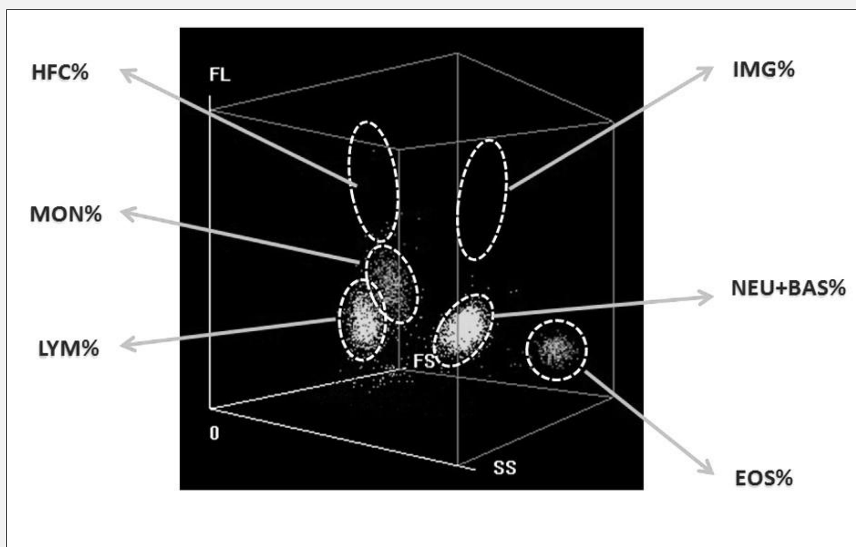

Figure 1. The FS/SS/FL dot plot shows the WBC differential and the measurement of the IMG parameter by the automated hematology analyzer.

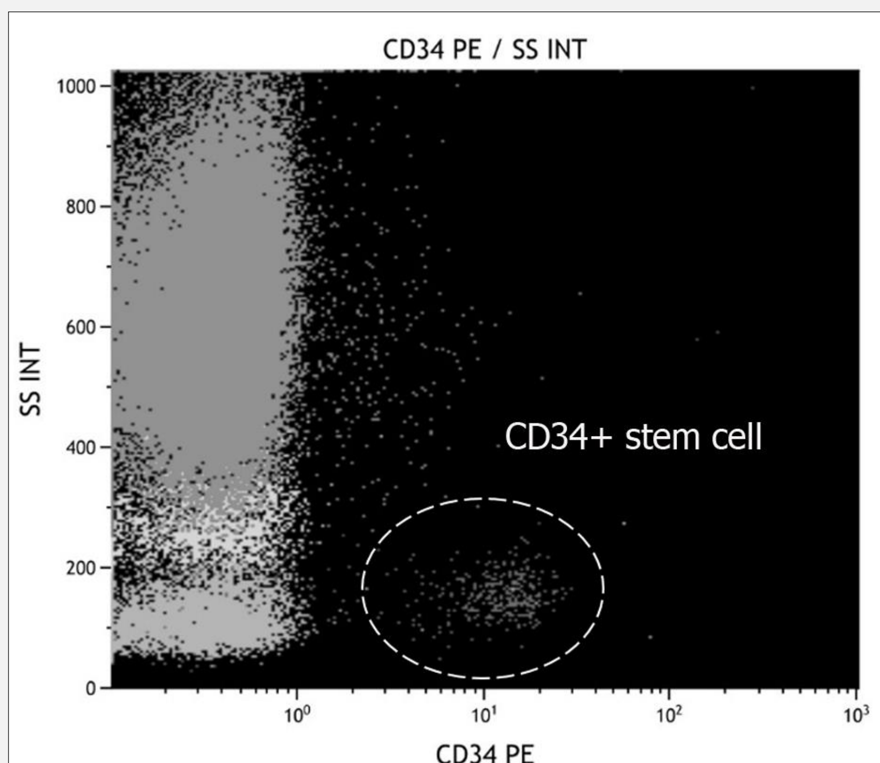

Figure 2. The SS/CD34 dot plot of the flow cytometric CD34+ stem cell enumeration.

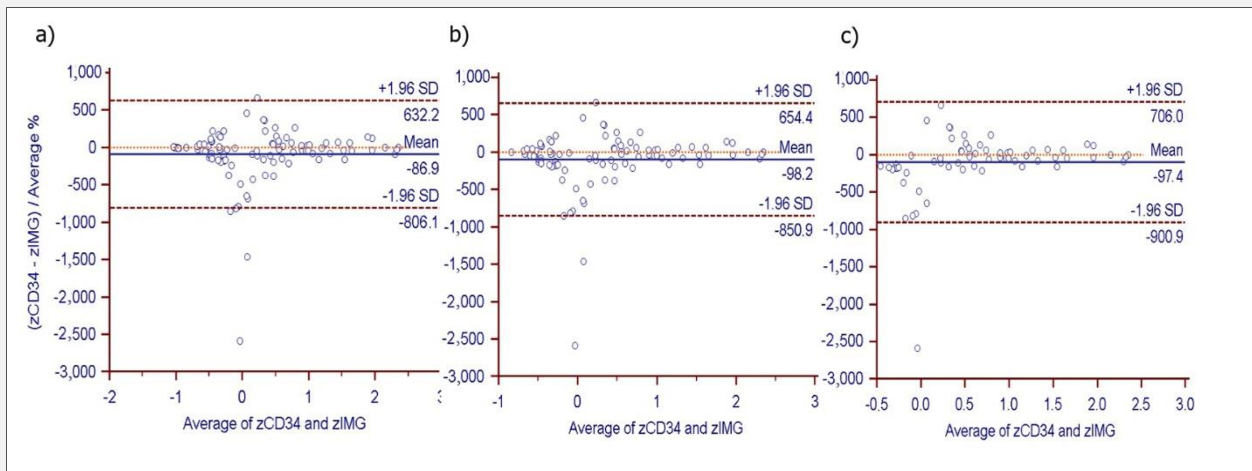

Figure 3. The agreement between the IMG counting by the hematology analyzer and the flow cytometric enumeration of CD34+ stem cells was evaluated by the Bland Altman analysis using the cutoff values of a) > 20/µL, b) > 50/µL, and c) all samples separately.

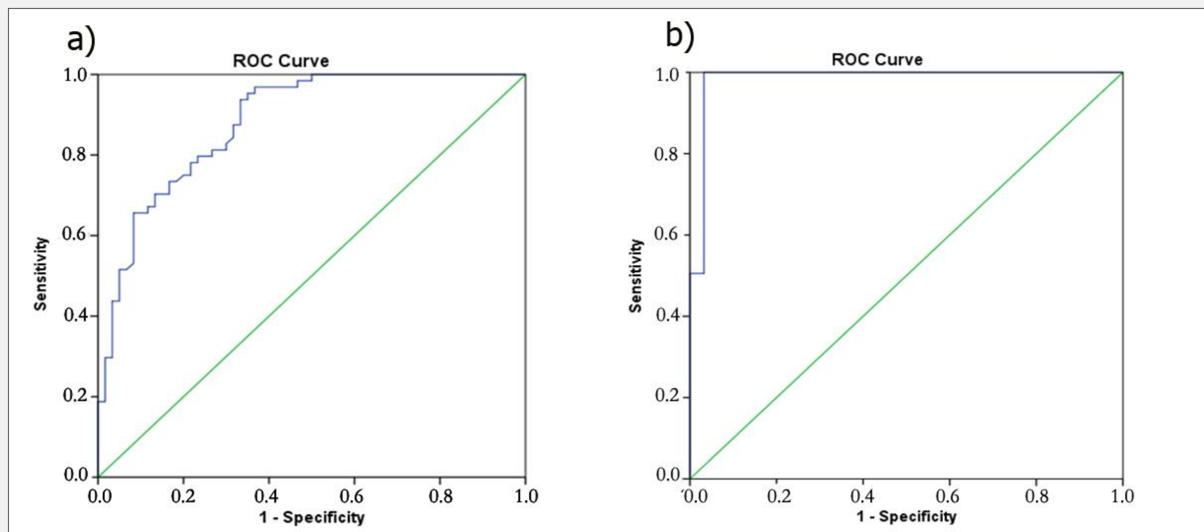

Figure 4. Methods´ sensitivity and specificity for CD34+ stem cells were determined by using ROC curves at different cutoff values.

When the cutoff value of 50/µL was taken, the sensitivity was 93.75 (84.8 - 98.3) and the specificity was 66.67 (53.3 - 78.3) for the IMG count of > 1.36/µL (a). When the cutoff value of 20/µL was taken for the CD34+ stem cell counting, the sensitivity was 100 (96.1 - 100) and the specificity was 96.77 (83.3 - 99.9) for the IMG count of > 0.09/µL (b).

DISCUSSION

The collection of the peripheral blood CD34+ stem cells by apheresis is an important resource in HSCT. Flow cytometric enumeration of CD34+ cells in peripheral blood is a validated method for the quantification of stem cells. However, it has disadvantages, such as high cost, technical difficulties, and long run times. It has been reported in the literature that the number of stem cells was low in 39% of the patients before apheresis, resulting in an insufficient yield on the first harvest [7]. This leads to ineffectual flow cytometric measurements. Hematology analyzers can detect hematopoietic precursors, including immature granulocytes. Furthermore, they have advantages such as lower costs, ease of use, and short-run times. This study investigated the feasibility of the use of immature cell counting by a hematology analyzer in the selection of samples for the flow cytometric CD34+ stem cell enumeration to be performed before leukapheresis.

A study showed that there is a correlation between the peripheral blood WBC counting by the hematology analyzer and the CD34+ stem cells enumeration by flow cytometry, using the same sample for the analysis [11]. In another study on apheresis products, CD34+ stem cell counts were reported to be correlated with the WBC and immature cell counts measured by the XE2100 hematology analyzer (Sysmex Co. Kobe, Japan) [14]. In our study, IMG counting on the hematology analyzer and the flow cytometric enumeration of CD34+ stem cells were performed simultaneously on 124 peripheral blood samples, and the results were compared. A significant correlation was found between the peripheral blood IMG counts and the flow cytometric enumeration of CD34+ stem cells ($r = 0.71$, $p = 0.000$) (Table 2). The WBC parameter used in previous studies indicates the total number of mature and immature leukocytes in the peripheral blood. However, the MINDRAY BC 6200 hematology analyzer quantifies the IMG specifically and identifies immature cells within the WBCs. Therefore, it can yield better indicative results compared to WBC count parameter alone. In this study, the Bland Altman analysis was performed to show the agreement between the two methods. A significant agreement was found at all concentrations (Figure 3). However, it is recommended that the CD34+ stem cell count should be > 20/µL in order to collect sufficient apheresis products. Values in the range of 10 - 20/µL are considered to be in the gray zone, while values < 10/µL indicate a failed leukapheresis procedure. Flow cytometric CD34+ stem cell enumeration in the peripheral blood is of major importance in demonstrating that optimum mobilization has occurred. However, optimum mobilization does not always occur in donors or patients; in particular, insufficient mobilization is observed in patients with a history of previous chemotherapy [15]. This results in ineffectual flow cytometric analysis of samples containing a CD34+ cell count of < 20/µL, and thus increases costs and causes loss of labor and time. Therefore, in this study, the correlations between IMG and CD34+ stem cell values were evaluated for the cutoff values of 20/µL and 50/µL, separately. Accordingly, when the cutoff value of > 20/µL was taken for the CD34+ number; there was a correlation between the two methods (Table 2). The Bland Altman analysis showed that there was an agreement between the two methods at the same cutoff value (Figure 3). However, no correlations were observed at cutoff values of < 20/µL. A ROC analysis was performed to determine the IMG count that can be used in the selection of suitable donors. When a cutoff value of 20/µL was taken for the CD34+ stem cell count, the highest sensitivity and specificity values were found in the presence of an IMG count of 900/µL (Figure 4). Accordingly, the flow cytometric enumeration of CD34+ stem cells can be planned for the peripheral blood samples with an IMG count of > 900/µL cells. Thus, ineffectual flow cytometry measurements can be avoided in samples containing IMG less than 900/µL.

In the literature, there are studies conducted on hematology analyzers measuring HPC [16-19]. However, the HPC parameter is measured only in some hematology analyzers and identifies immature blast cells that constitute a subgroup of IMG. Thus, the HPC parameter can be used to identify cells more specifically compared to the identification of only IMG. Studies report correlations ranging from 0.45 to 0.81 between HPC and CD34+ stem cell counts. A study reported that although a good correlation was found between the HPC measurement and the CD34+ cell count in the peripheral blood, a weak correlation ($r = 0.464$) of the HPC count was observed in the collection product [20]. In another study, Vogel et al. reported similar results [20,21]. In those studies, it was suggested that the HPC parameter at specified cutoff values can be used as an alternative directly to the flow cytometric CD34+ stem cell enumeration. However, they emphasized the necessity of flow cytometric CD34+ stem cell enumeration outside of these cutoff values. In this study, we suggest that the IMG counting by the hematology analyzer can be used not as an alternative to the CD34+ stem cell enumeration but in the pre-determination of samples for CD34+ stem cell enumeration by flow cytometry. This is because both IMG and HPC counting methods can be used to identify immature cells, but stem cell-specific antibodies are not used in any of these methods. In addition, studies have found differences ranging from 2 to 4 times between the means of HPC and CD34+ stem cell counts [6,10]. In other words, there is insufficient evidence that the analysis with HPC parameters can replace the flow cytometric CD34+ stem cell enumeration. The data collected in the hemogram analysis are used in the IMG counting method proposed in this study. Therefore, it does not incur further costs and requires additional sampling in routine studies.

CONCLUSION

The cutoff value of 900/µL can be used for the IMG counting by Mindray BC 6200. The IMG counting cannot replace the flow cytometric CD34+ stem cell enumeration but can be used as a preliminary test before the flow cytometric stem cell enumeration.

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Declaration of Interest:
The authors have no conflicts of interest to declare.

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