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Flow cytometric enumeration of CD34$^+$ hematopoietic stem and progenitor cells in leukapheresis product and bone marrow for clinical transplantation: a comparison of three methods

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Abstract: Flow cytometric enumeration of CD34$^+$ hematopoietic stem and progenitor cells (HSCs) is widely used for evaluation of graft adequacy of peripheral blood and bone marrow stem cell grafts. In the present study, we review and compare the major counting techniques of stem and progenitor cells. The methods are: the Milan/Mullhouse protocol, two-platform ISHAGE (International Society of Hematotherapy and Graft Engineering) and single-platform ISHAGE analysis system. According to the Milan/Mulhouse protocol, HSCs are identified by CD34 antibody staining and easy gating strategy. The ISHAGE guidelines for detection of CD34$^+$ cells are based on a four-parameter flow cytometry method (CD34PE/CD45PerCP staining, side and forward angle light scatter) thus employing multiparameter gating strategy. With two-platform ISHAGE protocol, an absolute CD34$^+$ count is generated by incorporating the leukocyte count from an automated hematology analyser. The single-platform ISHAGE method to determine the absolute CD34$^+$ count directly from a flow cytometer includes the use of Trucount tubes (Becton Dickinson) with a known number of fluorescent beads. CD34$^+$ cells were quantified in mobilized peripheral blood, collected by leukapheresis, and bone marrow from 42 samples from patients with hematological malignancies. The differences against the means display low disagreement between the Milan/Mulhouse and ISHAGE protocols, with discrepancies of up to 2.5% (two-platform ISHAGE) - 2.6% (single-platform ISHAGE) in enumeration of CD34$^+$ cells in leukapheresis product and 4.8% (two-platform ISHAGE) - 4.9% (single-platform ISHAGE) in bone marrow. Our results show high correlation among all three methods. Since the three protocols are compatible, choosing the most convenient in terms of costs, simplicity and compliance with clinical results appears to be a logical consequence. (www.cm-uj.krakow.pl/FHC)

Key words: CD34 - Hematopoieic stem cells - Enumeration - Flow cytometry

Introduction

Hematopoietic stem and progenitor cells (HSCs) obtained either from bone marrow (BM) or mobilized peripheral blood (PB) are used for autologous transplantation following high-dose chemotherapy in patients with hematological malignancies [4, 11, 12] . These cells are characterized by the expression of the adhesion receptor CD34 [13, 22, 23, 25]. The clinical value of determination of the accurate absolute numbers of CD34$^+$ cells is the most important parameter for evaluating stem and progenitor cell content in hematopoietic transplantation [6, 7, 9].

In this regard, several assays for CD34$^+$ cell enumeration have been proposed, but lack of standardized procedures has resulted in the generation of divergent data [15-18, 20, 24]. The first attempt at enumerating CD34$^+$ progenitors by flow cytometry was Milan/Mulhouse protocol based on the evaluation of CD34$^+$ cells with a low light scatter (SSC), after initial forward vs SSC live gating excluding debris, platelets, erythrocytes and cell aggregates [5,17]. This protocol is based on two-platform method, whereby the percentage of CD34$^+$ cells is

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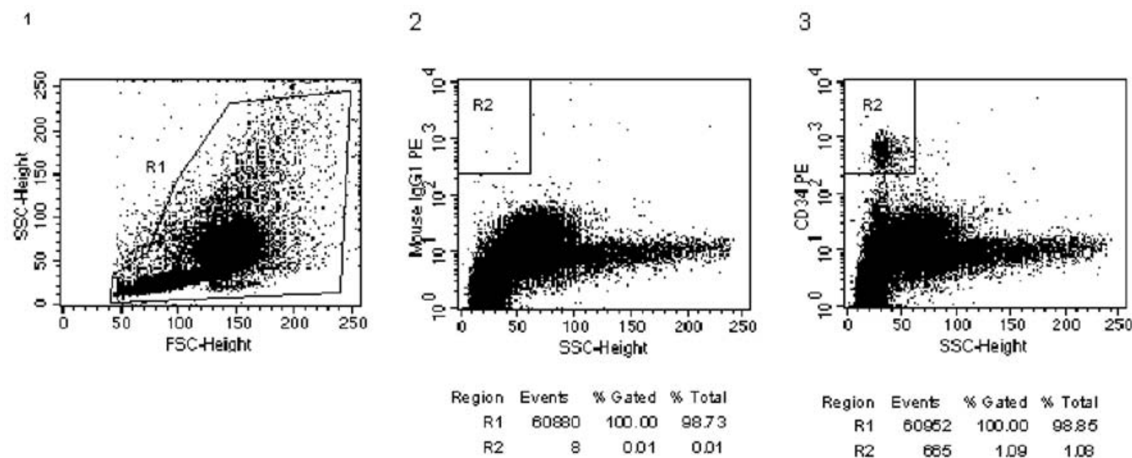


Fig. 1. Milan/Mulhouse gating strategy on a leukapheresis sample.

determined by flow cytometry and leukocyte count by an automated hematology analyzer.

Most laboratories propose the second two-platform method - ISHAGE protocol published by the International Society of Haematotherapy and Graft Engineering, designed as a set of guidelines for the accurate detection of $CD34^+$ cells based on four-parameter flow cytometry method (CD45PerCP/CD34 PE staining, side and forward angle light scatter) [19, 20]. Importantly, this approach allows the discrimination of HSCs (which express relatively low levels of CD45 on their surface) from lymphocytes and monocytes, and thus allowing the verification of "true" $CD34^+$ cells as being dim for CD45 fluorescence and having low side scatter ($CD45^{dim}$, SSC^{low}) [19, 21].

The development of single-platform methods enable the absolute $CD34^+$ cell count to be determined by a single instrument - flow cytometer [2, 5]. The flow cytometry based on single-platform method concentrates on incorporation of a known number of fluorescent beads into the sample. Using a lyse-no-wash procedure for sample preparation enables the ratio of $CD34^+$ cells to beads to be determined and the absolute $CD34^+$ cells count to be calculated. One of them is the single-platform ISHAGE [3, 10]. This new protocol combines the advantages of Trucount tubes and the ISHAGE gating strategy.

In the present study, we compare all three protocols: two-platform protocols (ISHAGE and Milan/Mulhouse) and single-platform ISHAGE protocol with each other.

Materials and methods

Cell sources. Bone marrow (BM) and leukapheresis product (LKP) samples were collected from 42 patients with hematological malignancies. BM aspirates (n=10) were harvested from patients in steady-state hematopoiesis undergoing general anesthesia for routine surgical procedures. LKP samples (n=32) were collected following chemotherapy and mobilization with subcutaneous granulocyte colony-stimulating factor (G-CSF, filgrastim) at 10 µg/kg per day. The leukaphereses were initiated when the number of $CD34^+$ cells was $\geq 20\times10^6$/l and performed as an outpatient procedure on a Fenwal CS-3000 Plus (Baxter) separator with a continuous flow of blood cells following the manufacturer's instruction for mononuclear cell collection.

Monoclonal antibodies (MoAbs). The following mouse-derived antibodies were used in this study: phycoerytrin (PE)- conjugated 8G12 (HPCA-2), peridinin-chlorophyll-protein (PerCP)-conjugated CD45 (2D1), PE-IgG1 isotype control; antibodies were purchased from Becton Dickinson (BD).

Cell preparation and immunofluorescence staining. For each patient's sample, five tubes were prepared and processed in parallel: (1) single staining with CD34 PE were added to the wash tube; (2) double staining with CD34 PE and CD45 PerCP - wash tube; (3) double staining with CD34 PE and CD45 PerCP - Trucount tube; (4) isotype control reagent-IgG1 PE and CD45 PerCP - wash tube and (5) isotype control reagent-IgG1 PE and CD45 PerCP - Trucount tube. When necessary, the samples were diluted to adjust the leukocyte count to 10×10^9/l and 100 µl aliquots were placed onto the bottom of the tubes. To block binding of Fc receptors and avoid to nonspecific staining, the cells were subsequently incubated with 10% (vol/vol) heat-inactivated human AB serum at 4°C. Tubes were stained and prepared according to the manufacturer's recommendation. In case of single-platform method (lyse/no wash) cells were not washed (tubes no. 3 and no. 5). All samples were then stored at 4°C in the dark and analysed by flow cytometry within 1 h.

Hematological cell counts. The total number of $CD34^+$ cells from BM and LKP samples were measured by an automatic cell counter, the Sysmex F820 (TOA Medical Electronics Kobe, Japan) or by direct flow cytometer (Trucount BD). In case of two-platform methods, the results were expressed as %$CD34^+$ cells, and the absolute $CD34^+$ cells/µl calculated as %$CD34^+$ cells × WBC×10^3µl. In case of single-platform method, the number of $CD34^+$ cells per microliter was calculated according to the following formula:

$$CD34^+ \text{ cells/}\mu l = \frac{\text{No. of } CD34^+ \text{ cells} \times \text{bead count per test} \times \text{dilution factor}}{\text{No. of beads collected}}$$

Sample acquisition. Cells were acquired on three-color FACS Calibur flow cytometer (BD) equipped with a 488 nm argon laser and analyzed with CellQuest 3.1 software. The instrument was aligned

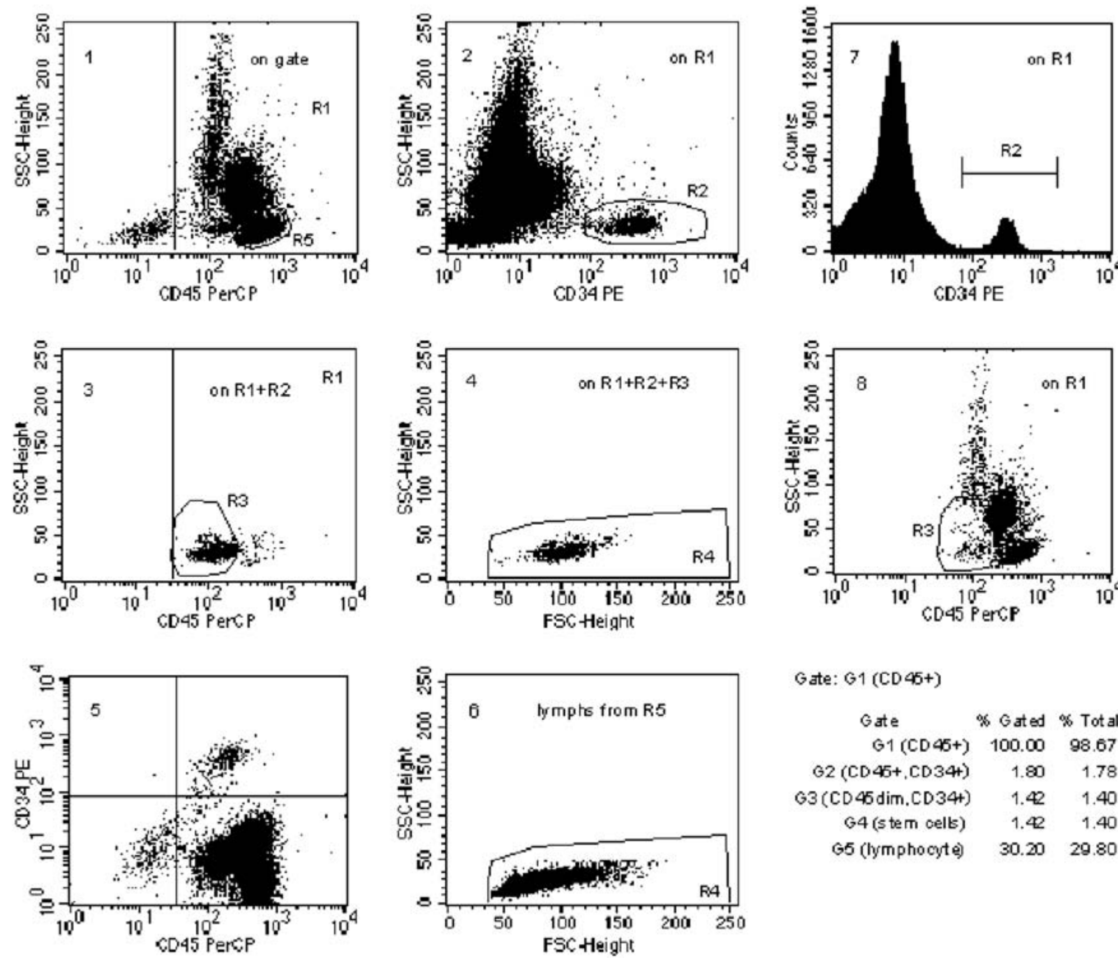


Fig. 2. Two-platform ISHAGE strategy on a leukapheresis sample. **1:** Sample stained with anti-CD45 PerCP. **2:** Anti-CD34 PE staining of cells in region R1. Region R2 represents CD34$^+$ cells. **3:** CD45 vs. side-scatter analysis of CD34$^+$ cells gated by R1 and R2. **4:** Region R4 represents a blast/lymphocyte region (cells gated by R1, R2 and R3). **5:** CD45 vs. CD34 analysis of all events. **6:** Region R5 - lymphocytes. **7:** CD34$^+$ cells enumerated only by gating the positive cells in region R1. **8:** CD45 vs. side-scatter analysis of events gated in region R1.

and calibrated daily using a three-color mixture of CaliBRITE beads (BD) with FACSComp software (BD). In order to comply with statistical requirements, 50 000 events were acquired for each sample in Milan/Mulhouse protocol. In the event of ISHAGE (single- and two-platform) a total of 100 000 leukocytes (G1), and in case of single-platform ISHAGE 2 000 bead events were collected.

Gating strategy: Milan/Mulhouse protocol. According to the Milan/Mulhouse protocol, nucleated cells were identified and distinguished from platelets, unlysed erythrocytes, and debris by their higher FSC signals (R1 in Fig. 1, Plot 1). The FL vs. SSC plot of the isotype control mAb-stained sample was then used to set the marker discriminating between positive and negative FL signals (R2 in Fig. 1, Plot 2). Using this marker, HCSs were identified as CD34$^+$SSC^{low} in the CD34 mAb-stained sample (R2 in Fig. 1, Plot 3). Finally, the events in R2 of the isotype control (Fig. 1, Plot 2) were subtracted from the number of CD34$^+$ events (Fig. 1, Plot 3). The basis was nucleated cells (as based on FSC and SSC criteria) and absolute counts were derived from a separate leukocyte count on a hematology analyzer.

Gating strategy: two-platform ISHAGE protocol. For ISHAGE analysis, a region R1 (Fig. 2 and Fig. 3, Plot 1) representing CD45$^+$ events was established to include all nucleated WBCs. Red blood cells, nucleated red blood cells, platelets, and other cellular debris are excluded from this region since they do not express CD45 structures. CD45$^+$ events in the region R1 were then analyzed for CD34 staining, and positive events were gated into R2 (Fig. 2 and Fig. 3, Plot 2). Events defined by regions R1 and R2 were analyzed on dot-plot describing granularity vs. CD45 staining. Into the region R3 were gated events representing true blast cells, which were identified as SSC^{low}, CD45^{dim} (Fig. 2 and Fig. 3, Plot 3). Events into region R4 were defined as cells with characteristics of blast cells and lymphocytes. Cells from region R4 were named true CD34$^+$ blast cells (Fig. 2 and Fig. 3, Plot 4). The region R5 contained lymphocytes (CD45^{bright}, SSC^{low}) (Fig. 2 and Fig. 3, Plot 5).

Gating strategy: single-platform ISHAGE protocol. Figure 4 shows the gating strategy for CD34 analysis using Trucount tubes. Since Trucount beads produce a very low forward scatter signal, a forward scatter threshold cannot be used. A FL3 threshold was set on CD45 expression, with care to ensure that CD45^{low}/ CD34$^+$ cells were not excluded from the analysis (Fig. 4, Plot 1). The Trucount beads, evident as a population of bright events in each fluorescence channel, were enumerated in region R6 and R7 (Fig. 4, Plot 5 and Plot 7). To exclude nonbead events, R6 was drawn tightly around the beads in line with the manufacturer's instruction. CD34$^+$ events were enumerated in G4 (Fig. 4, Plot 4).

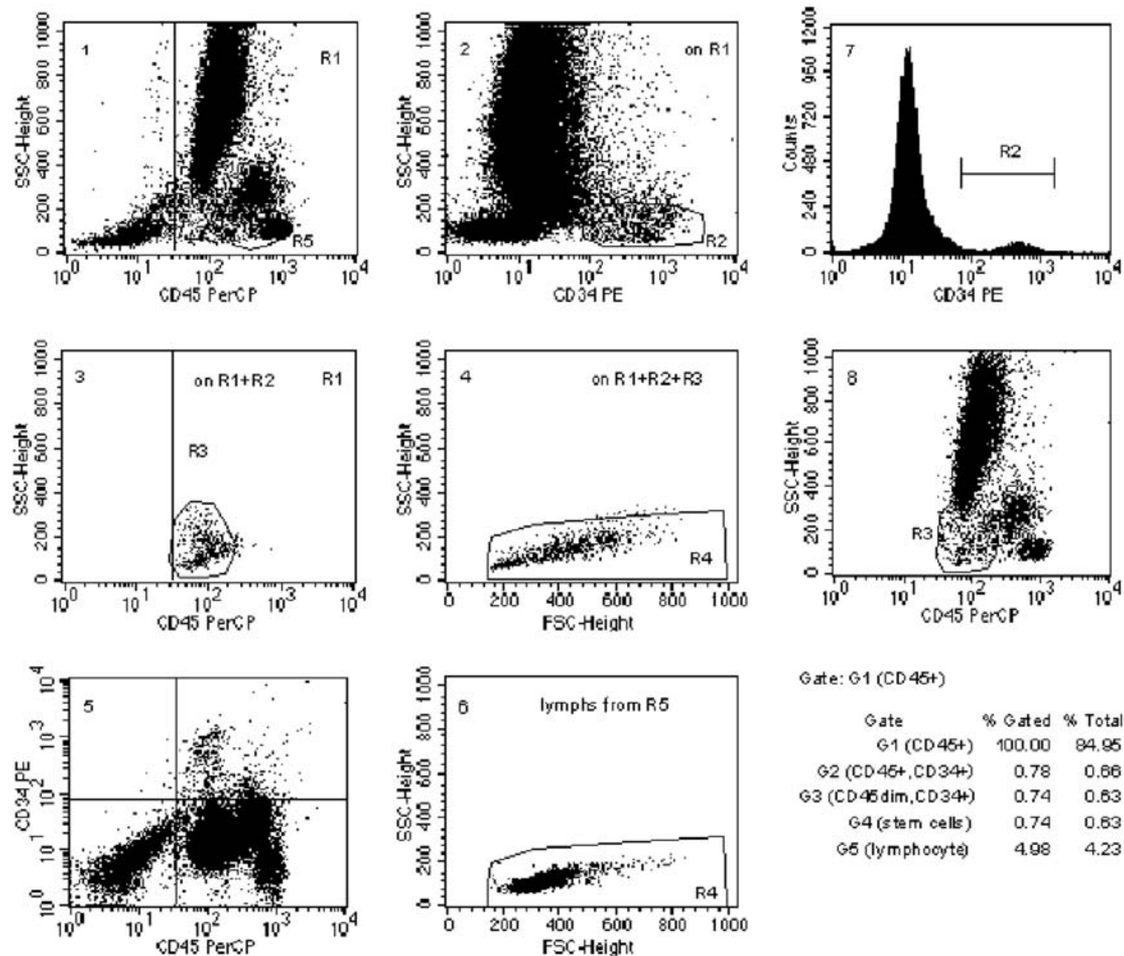


Fig. 3. Analysis of $CD34^+$ cells in BM. Analysis was performed exactly as described for the LKP sample in Figure 2.

Evaluation of linearity and reproducibility. For the linearity assay, serial dilutions were prepared, starting from 1000, 500, 150, 50 $CD34^+$ cells/µl and quantified by the three different methods. Precision of the absolute counting method was measured at three target points. Samples with approximately 1000, 150, and 50 $CD34^+$ cells/µl were processed two times for each method.

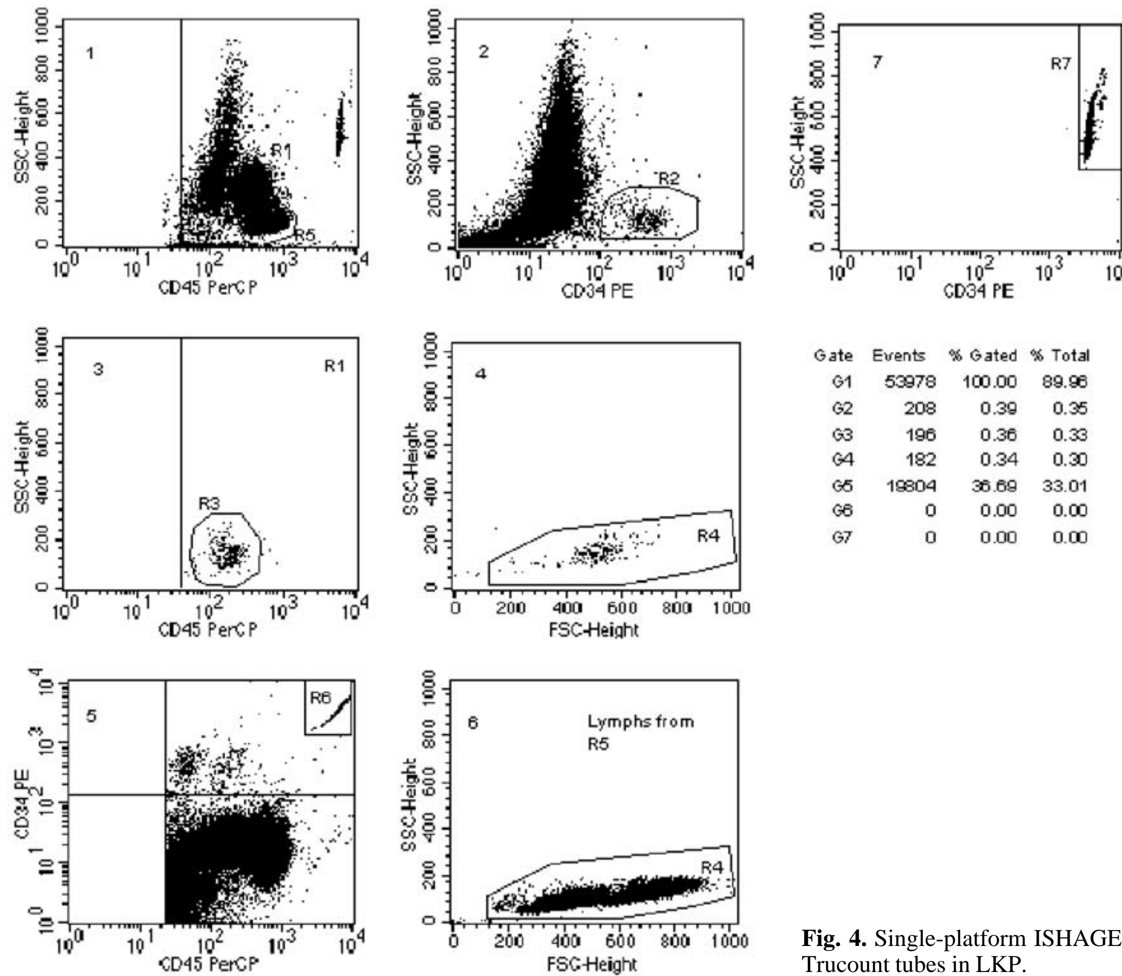
Statistical analysis. The results obtained by each of the three methods were compared using the Wilcoxon matched pairs test for nonparametrically distributed data, and linearity regression analysis, using Statistica software. Agreement between the methods was assessed by analysis of the relationship between the differences and the mean of the differences as recommended by Bland and Altman (MedCalc statistical software). Coefficients of variation (CV) were calculated according to the following formula:

[standard deviation (SD)/mean] × 100(%)

Results

The analysis of the 42 samples tested showed no significant differences for the results obtained by the Milan/Mulhouse protocol in comparison to single-platform ISHAGE and two-platform ISHAGE methods. Linear regression analysis for Milan/Mulhouse vs. ISHAGE two-platform and vs. ISHAGE single-platform gave respectively $r = 0.96$ and $r = 0.95$ (Table 1). Statistical analysis of $CD34^+$ population demonstrated that results obtained by single-platform ISHAGE assay showed the highest correlation with the two-platform ISHAGE assay, giving $r = 0.98$. The Wilcoxon signed rank test showed that there was no statistically significant difference between the three methods ($p > 0.05$).

Reproducibility was tested by replicate measurements of samples with known $CD34^+$ cell concentrations, and results were expressed as a coefficient of variation (CV) (Table 2). For the lowest sample concentration of 50 $CD34^+$cells/µl, CV values were the highest, being 12.2% for the Milan/Mulhouse, 4.3% for the two-platform ISHAGE and 4.0% for the single-platform ISHAGE assays. For higher cells concentration (150 and 1000 $CD34^+$cells/µl), CV values for single-platform assay were similar to those for the two-platform assays, proving comparable precision of the three methods (Table 2). Finally, in order to further explore whether the methods differ from each other, we used a statistical approach aimed at assessing the degree of compatibility.



Gate	Events	% Gated	% Total
G1	53978	100.00	89.96
G2	208	0.39	0.35
G3	196	0.36	0.33
G4	182	0.34	0.30
G5	19804	36.69	33.01
G6	0	0.00	0.00
G7	0	0.00	0.00

Fig. 4. Single-platform ISHAGE gating strategy using Trucount tubes in LKP.

Our results (Fig. 5) showed a graphical representation of differences between the tested methods against their mean, to take into consideration the sources of HSCs. Bland-Altman plot (Fig. 5) revealed the majority of data points distributed around the mean of the three methods. In line with the results of regression analysis, the methods are indeed comparable since considerable discrepancies do not exist and degree of compatibility is acceptable (disagreement = 2.5% in Milan/Mulhouse vs. ISHAGE two-platform protocol, 2.6% in Milan/Mulhouse vs. ISHAGE single-platform in case of LKP, and disagreement = 4.8% in Milan/Mulhouse vs. ISHAGE two-platform protocol and 4.9% in Milan/Mulhouse vs. ISHAGE single-platform protocol in case of BM). However, using the ISHAGE analysis, the CD34$^+$ cells from bone marrow in region R2 exhibited a more heterogeneous staining pattern than that of the leukapheresis sample (Fig. 3 Plot 7 and Fig. 2 Plot 7). Additionally, these BM CD34$^+$ cells showed a greater range of granularity when analyzed on a dot-plot describing CD45 fluorescence vs. side scatter (Fig. 3 Plot 3, region R3). In such case, where the bone marrow may be enriched with primitive cell types (as a consequence of post-chemotherapy bone marrow regeneration), it might be more useful to enumerate CD34$^+$ cells in the R3 region rather than in the R4 region.

Discussion

Flow cytometric enumeration of CD34$^+$ cells has become widely accepted as the technique of choice to quantify HSCs for the clinical management of stem cell transplantation [8, 15, 20].

Despite the establishment of guidelines for CD34$^+$ cell enumeration by two-platform flow cytometric assay, standardization of results has been difficult to achieve [14, 15, 19, 20].

In order to contribute to this issue we have compared three different methods of CD34$^+$ cell enumeration: the Milan/Mulhouse protocol, the two-platform ISHAGE protocol and single-platform ISHAGE protocol with the aim of verifying the differences between them, especially in terms of clinical impact.

The new simplified single-platform assay should avoid some sources of the probable errors involved in the two-platform assay, namely the washing step after

Table 1. Regression analysis between the three methods

Methods	N	R	P-value
Milan-Mulhouse / ISHAGE two-platform	42	0.96	<0.001
Milan-Mulhouse / ISHAGE single-platform	42	0.95	<0.001
ISHAGE two- / ISHAGE single-platform	42	0.98	<0.001

Table 2. Reproducibility of the same sample tested four times

Sample concentration (CD34+ cells/µl)	Coefficient of variation (%)		
	Milan/ Mulhouse	ISHAGE two-platform	ISHAGE single-platform
50	12.2	4.3	4.0
150	6.4	3.6	3.2
1000	3.1	2.9	2.6

staining and the use of the white blood cell count for calculation of CD34+ cell concentration. Adding a known number of fluorescent beads to the single-platform ISHAGE protocol allows the determination of absolute CD34 counts without the added variable of hematology analyzer. In fact, this method has the advantage of being rapid and straightforward. This advantage of single-platform assay offers the possibility of higher level of standardization between laboratories. However, comparison of the two-platform ISHAGE protocol with the single-platform ISHAGE protocol showed a high degree of concordance over a wide range

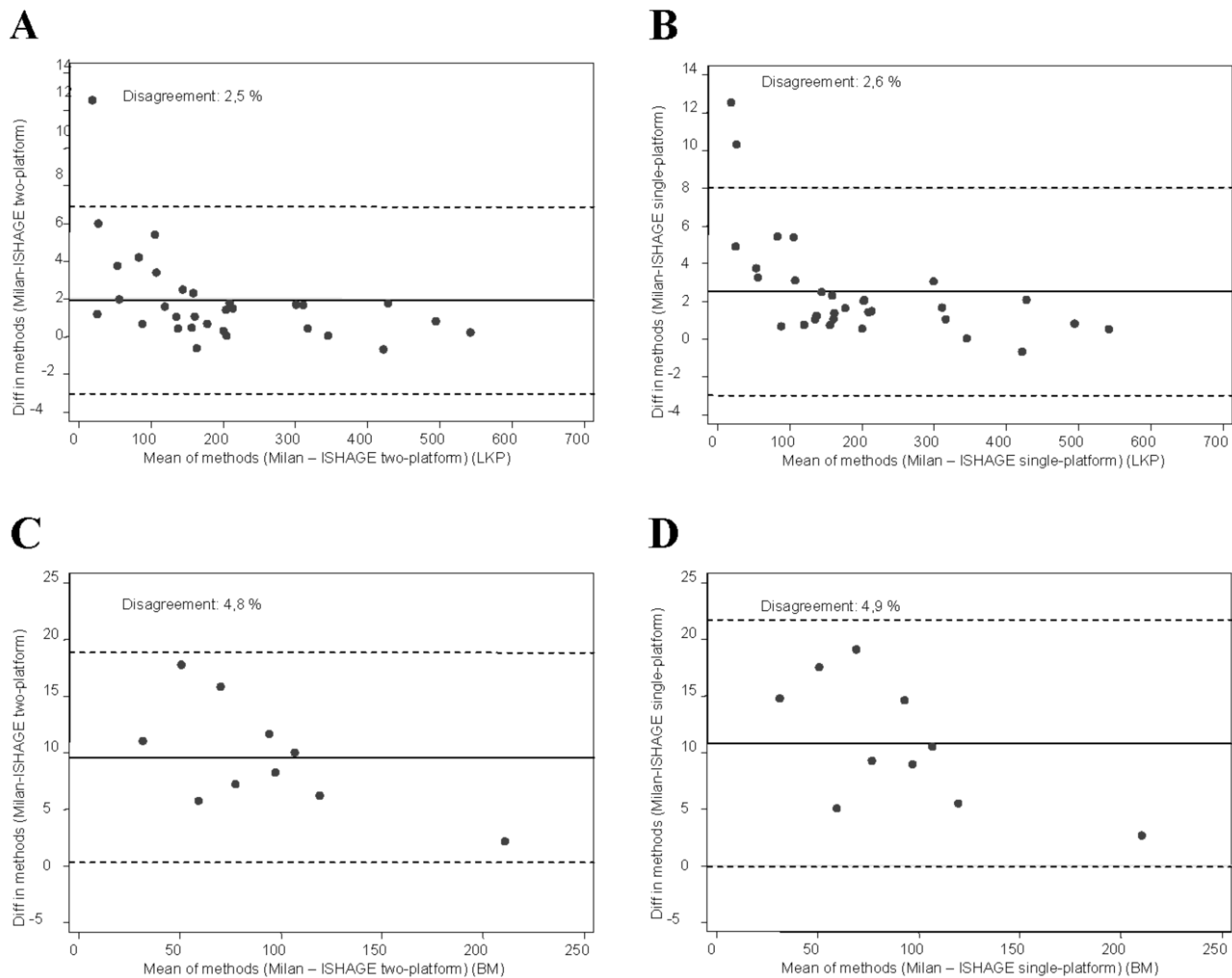


Fig. 5. Bland-Altman plot displaying differences of methods against their mean. **A:** Milan/Mulhouse vs. two-platform ISHAGE in LKP. **B:** Milan/Mulhouse vs. single-platform ISHAGE in LKP. **C:** Milan/Mulhouse vs. two-platform ISHAGE in BM. **D:** Milan/Mulhouse vs. single-platform ISHAGE in BM. Solid line represents the mean of differences or bias. Dashed lines represent mean of the difference (bias) ± 2 SD. A mean bias equal to zero represents perfect agreement.

of CD34 counts (correlation coefficient r = 0.98). Our results are consistent with those of Keeney *et al.* [10], but Brocklebank and Sparrow [1] reported significant disagreement between the methods. However, the latter study concerned cord blood.

For each of the methods, linearity was observed, with a good relationship between the results as demonstrated by the high correlation coefficient (Table 2). The precision of the methods evaluated by replicate measurements was reflected by low CV values for the all the concentrations tested, with the exception of the Milan/Mulhouse assay for the lowest concentration of $CD34^+$ cells (50/µl). These high CV values may be associated with the detection level of this method and gating strategy.

In terms of gating strategy, in the Milan/Mulhouse protocol the major contaminants appeared to be red blood cells and their nucleated precursors, based on their light-scattering characteristics (very low forward scatter and low side scatter). Red blood cells, their nucleated precursors, platelets and other cell debris than can inflate the number of $CD34^+$ events, and which are present in highly variable numbers in clinical samples, are thereby excluded. In case of the ISHAGE assays, lymphocytes that exhibit characteristics of side and forward scatter similar to that of true $CD34^+$ blasts can now be separated from $CD34^+$ cells since they stain brightly for CD45. Similarly, monocytes, which can partially overlap blast/lymphocytes gates on light-scatter analysis, can be distinguished by their high CD45 expression and increased side scatter.

Our results showed that the studied methods are comparable and therefore interchangeable due to a very low degree of disagreement. The value of disagreements is higher for evaluation of $CD34^+$ from bone marrow than from leukapheresis product, when comparing the Milan/Mulhouse protocol with the single-platform ISHAGE and two-platform ISHAGE protocol. Comparison of $CD34^+$ cells from leukapheresis product and bone marrow showed that bone marrow $CD34^+$ cells exhibited a heterogeneous staining pattern as well as heterogeneous granularity.

In summary, comparison of the Milan/Mulhouse and the single-platform ISHAGE techniques with our routine two-platform ISHAGE flow cytometric assay gave high correlation. The new method, single platform ISHAGE, allows the direct determination of absolute numbers of $CD34^+$ cells. However in our hands, comparison of the two-platform ISHAGE protocol with modified single-platform ISHAGE protocol showed high correlation and compatibility. The sequential gating strategy of the ISHAGE protocol requires more manual work as compared to the Milan/Mulhouse protocol, but undoubtedly is a more sensitive and reproducible method of enumerating $CD34^+$ cells derived from bone marrow and mobilized peripheral blood.

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