



## Relative frequency of immature CD34+/CD90+ subset in peripheral blood following mobilization correlates closely and inversely with the absolute count of harvested stem cells in multiple myeloma patients

Relativna učestalost nezrelog podtipa ćelija CD34+/CD90+ u perifernoj krvi posle mobilizacije je u tesnoj i obrnutoj korelaciji sa apsolutnim brojem matičnih ćelija u afereznom produktu kod bolesnika sa multiplim mijelomom

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### Abstract

**Background/Aim.** Stem cells (SCs) guarantee complete/long-term bone marrow (BM) repopulation after SC-transplants. The aim of the study was to evaluate absolute count of total SCs (determined by ISHAGE-sequential-gating protocol – SC<sup>ish</sup>) and relative frequency of immature CD34+/CD90+ (CD90+SC<sup>ish</sup>) subset in peripheral blood (PB) as predictive factors of mobilization and apheresis product (AP) quality. **Methods.** Mobilization included chemotherapy and granulocyte-growth-factor (G-CSF). Harvesting was performed by Spectra-Optia-IDL-system. The SC<sup>ish</sup> were determined as a constitutional part of CD34+ cells in the “stem-cell-region” using FC-500 flow-cytometer. In this study, the original ISHAGE-sequential-gating protocol was modified by introduction of anti-CD90-PE monoclonal-antibody into the analysis of CD90 expression on SC<sup>ish</sup> (CD90+SC<sup>ish</sup>). The results were presented as a percentage of SC<sup>ish</sup> per nucleated-cell count, absolute SC<sup>ish</sup> count in  $\mu$ L of the PB or the AP, percentage of the CD90+SC<sup>ish</sup> expressed to SC<sup>ish</sup> and absolute CD90+SC<sup>ish</sup> count in  $\mu$ L of the PB or the AP. **Results.** The absolute count of total SC<sup>ish</sup> and CD90+SC<sup>ish</sup> was significantly higher ( $p = 0.0007$  and  $p = 0.0266$ , respectively) in the AP than in the PB samples. The CD90+SC<sup>ish</sup>/total SC<sup>ish</sup> indexes from PB were higher than indexes from the AP ( $p = 0.039$ ).

### Apstrakt

**Uvod/Cilj.** Matične ćelije (MĆ) obezbeđuju kompletnu/dugotrajnu repopulaciju kostne srži (KS) posle trans-

The relative frequency of CD90+SC<sup>ish</sup> showed a highly significant inverse correlation with the absolute count of total SC<sup>ish</sup> in both, the PB and AP ( $p = 0.0003$  and  $p = 0.0013$  respectively). The relative frequency of CD90+SC<sup>ish</sup> from the PB also showed a significant ( $p = 0.0002$ ) inverse relationship with total SC<sup>ish</sup> count in the AP. Patients with less than 10% CD90+SC<sup>ish</sup> in the PB had evidently higher ( $p = 0.0025$ ) total SC<sup>ish</sup> count in the AP. **Conclusion.** We speculate that lower CD90+SC<sup>ish</sup> yield in the AP is not a consequence of an inferior collection efficacy, but most likely a result of several still not fully resolved immature SC cytomorphological/biophysical features. Therefore, following the mobilization by chemotherapy G-CSF, some logical questions appear – whether we should follow the absolute count of total SC<sup>ish</sup>, or, whether we should test for relative frequency of CD90+SC<sup>ish</sup> prior to harvesting. To reach the final conclusions, it is essential to conduct further controlled and larger investigations concerning the correlation of circulating and harvested SCs with patients' hematopoietic recovery.

### Key words:

stem cells; hematopoietic stem cell transplantation; bone marrow; flow cytometry; multiple myeloma; antineoplastic combined chemotherapy protocols.

plantacije. Cilj ove studije bila je procena apsolutnog broja ukupnih MĆ (utvrđena protokolom „ISHAGE-sequential-gating” – Stem-Cell<sup>ish</sup> [SC<sup>ish</sup>]) i relativne učestalosti primitivnih podtipova CD34+/CD90+ (CD90+SC<sup>ish</sup>) u perifernoj krvi (PK)

kao prediktora efikasnosti mobilizacije i pokazatelja kvaliteta afereznog produkta (AP). **Metode.** Mobilizacija je postignuta hemioterapijom/faktor-rasta-granulocitopoeze (G-CSF). Prikupljanje je izvedeno pomoću sistema Spectra-Optia-IDL. Čelije SC<sup>ish</sup> determinisane su kao konstitutivni deo CD34<sup>+</sup> u regiji-matičnih-ćelija („stem-cell-region“) upotrebom protočnog citometra FC-500. U ovoj studiji, originalni protokol „ISHAGE-sequential-gating“ modifikovan je uvođenjem monoklonskog antitela anti-CD90-PE radi analize ekspresije antigena CD90 na ćelijama SC<sup>ish</sup> (CD90<sup>+</sup>SC<sup>ish</sup>). Rezultati su prikazani kao procenat ćelija SC<sup>ish</sup> u odnosu na broj nukleisanih ćelija, apsolutni broj SC<sup>ish</sup> u  $\mu$ L PK ili AP, procenat ćelija CD90<sup>+</sup>SC<sup>ish</sup> izražen u odnosu na SC<sup>ish</sup> i apsolutni broj CD90<sup>+</sup>SC<sup>ish</sup> u  $\mu$ L PK ili AP. **Rezultati.** Apsolutni broj ukupnih ćelija SC<sup>ish</sup> i CD90<sup>+</sup>SC<sup>ish</sup> bio je značajno ( $p = 0,0007$  i  $p = 0,0266$ ) veći u uzorcima AP nasuprot PK. Indeks CD90<sup>+</sup>SC<sup>ish</sup>/ukupne SC<sup>ish</sup> u uzorku PK je bio veći od indeksa u uzorku AP ( $p = 0,039$ ). Relativna učestalost CD90<sup>+</sup>SC<sup>ish</sup> pokazala je vrlo značajnu inverznu korelaciju sa apsolutnim brojem ukupnih SC<sup>ish</sup> u PK i AP ( $p = 0,0003$  i  $p = 0,0013$ ). Relativna učestalost ćelija CD90<sup>+</sup>SC<sup>ish</sup> u PK takođe je pokazala značajnu

( $p = 0,0002$ ) inverznu korelaciju sa apsolutnim brojem ukupnih ćelija SC<sup>ish</sup>. Bolesnici sa manje od 10% CD90<sup>+</sup>SC<sup>ish</sup> u PK su imali značajno ( $p = 0,0025$ ) veći apsolutni broj ukupnih SC<sup>ish</sup> ćelija u AP. **Zaključak.** Smatramo da niži prinos CD90<sup>+</sup>SC<sup>ish</sup> u AP nije prouzrokovao manje efikasnim prikupljanjem, već je najverovatnije posledica različitih, još uvek samo delimično razjašnjenih, citomorfoloških/biofizičkih osobina manje zrelih MČ. Zato, posle mobilizacije hemioterapijom/G-CSF nameću se logična pitanja - da li bi trebalo pratiti apsolutni broj ukupnih SC<sup>ish</sup> ćelija ili je celishodnije testirati relativnu učestalost CD90<sup>+</sup>SC<sup>ish</sup> pre sprovođenja afereznog prikupljanja MČ. Za donošenje definitivnih zaključaka neophodna su buduća kontrolisana i sveobuhvatnija istraživanja MČ, u vezi sa utvrđivanjem korelacije cirkulišućih i priku-pljenih ćelija sa hematopoetskim oporavkom bolesnika.

#### Ključne reči:

ćelije, matične; transplantacija hematopoeznih matičnih ćelija; kostna srž; citometrija, protočna; multipli mijelom; lečenje kombinovanjem antineoplastika, protokoli.

### Introduction

The “cytopoiesis” – defined as *in vivo* cell development and expansion – is a multi-cyclic event in which a spectrum of mature cells is produced from a small number of stem-cells (SCs). SCs could be characterized as cells having well-balanced self-renewal, differentiation and proliferative capacity, as well as potential for plasticity, that is an ability to “switch” into other cell lineages. The SCs guarantee steady-state homeostasis in various “tissue-generating” (e.g. hematopoietic) systems<sup>1,2</sup>.

High-dose chemotherapy followed by allogeneic or autologous SC-transplants is considered as standard treatment for some malignant and few immune-mediated diseases (e.g. multiple sclerosis)<sup>1-3</sup>. The use of SCs for organ repair (damaged myocardium, liver, pancreas, etc) opens new perspectives in regenerative medicine<sup>1,3</sup>. For transplants, bone marrow (BM) has been the primary SC-source, in which approximately 2–4% of total nucleated cells (TNCs) express the CD34 antigen<sup>4,5</sup>. The CD34<sup>+</sup> cells were recognized in peripheral blood (PB), but in extremely low ratio in the “steady-state” hematopoiesis: 0.01–0.05% of the TNCs<sup>1,4</sup>. Mobilization by chemotherapy and recombinant human granulocyte-colony-stimulating factor (rHuG-CSF) radically increases the count of circulating CD34<sup>+</sup> cells numbers in patients and healthy donors<sup>1</sup>. However, just a small fraction of double positive (CD45<sup>+</sup>CD34<sup>+</sup>) cells, with typical size and specific intracellular granularity/complexity – according to the International Society for Hematotherapy and Graft Engineering (ISHAGE) protocol – represents “true” SCs (or SC<sup>ish</sup>)<sup>6-10</sup>. Moreover, immature or more primitive hematopoietic progenitors (PHPs) bare antigen CD90 (Thy-1), a 25 to 35 kDa molecule, which is also expressed by 1–4% of human fetal liver cells, umbilical cord blood (UCB), BM and several PB cells. PHPs are responsible for complete and long-term BM repopulation with durable or late hematopoe-

tic reconstitution – and are limited within the immature CD34<sup>+</sup>/CD90<sup>+</sup> subset (or CD90<sup>+</sup>SC<sup>ish</sup>)<sup>7,10-12</sup>. As confirmed, the CD34<sup>+</sup>/CD90<sup>+</sup> cells infused most appropriately predict platelet (Plt) recovery after the SC-transplants<sup>11</sup>. The CD34<sup>+</sup>/CD90<sup>+</sup> subset is also heterogeneous: evidently enriched in PHPs, but contains some less primitive lineage committed progenitors (LCPs). However, the majority of LCPs exist in an additional CD34<sup>+</sup>/CD90<sup>-</sup> (or CD90<sup>-</sup>SC<sup>ish</sup>) cell population<sup>7</sup>.

Traditional sources of the SCs are the BM and PB. The UCB has been used as an alternative source since the late 1980s<sup>1,13</sup>. Damages caused by the chemotherapy (applied prior to autologous SC-transplants) could be an important limiting factor of the SC-mobilization. Regularly, the count of total CD34<sup>+</sup> cells in PB of healthy donors is higher than in mobilized non-Hodgkin lymphoma patients<sup>12</sup>. However, after mobilization in PB of these patients the CD34<sup>+</sup> cell population is more immature, since they have a higher CD90 expression<sup>12</sup>. That could be a significant factor that influenced marrow repopulation, with special impact of late and durable hematopoietic reconstitution following SC-transplants<sup>11</sup>. This preclinical study aimed to evaluate absolute count of total SC<sup>ish</sup> (including the CD90<sup>+</sup>SC<sup>ish</sup> and CD90<sup>-</sup>SC<sup>ish</sup> subsets) and relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> in the PB, as predictive factors of the mobilization efficacy and of the apheresis product (AP) quality.

### Methods

In this pilot study the importance of our own novel predictive factors of the efficacy of the SC mobilization (absolute count of total SC<sup>ish</sup> and relative frequency of CD90<sup>+</sup>SC<sup>ish</sup>) using apheresis system Spectra-Optia IDL-system (Terumo-BCT, USA) were evaluated. Cell harvesting in a comparatively homogeneous (considering pre-transplant chemotherapy, mobilization protocol and conditioning regi-

men) category of multiple myeloma patients ( $n = 12$ ) were performed. Patients were aged 26–62 years; male/female ratio was 1.3 : 1. The study was performed according to the guidelines of the Declaration of Helsinki Principles and Good Clinical Practice and was approved by the local institutional Ethic board.

#### *Cell harvesting technology*

Standardized apheresis procedures – processing two patients' total blood volumes with equal quantity (200 mL) of the AP – were performed. The mobilization protocol included chemotherapy (cyclophosphamide 2–4g/m<sup>2</sup> and etoposide 400–800 mg/m<sup>2</sup>) with rHuG-CSF (12–16 µg/kgbm/day). Citrate-containing anticoagulant (Acid-Citrate-Dextrose, with 2.2% citrate concentration – ACD formula A, USP) was applied, at the same ACD : whole blood ratio (1 : 10) for all procedures. Additional patients' systemic or the AP heparinization was not performed. Vascular access was obtained across central venous catheter applied into subclavian or jugular and occasionally femoral veins. In this study, cell collections were performed when the absolute count of CD34<sup>+</sup> in the patient's PB was  $\geq 19.4 \pm 5 / \mu\text{L}$  and the relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> was  $\geq 9.3 \pm 12.2\%$ , respectively. The patients tolerated performed apheresis procedures well, without severe adverse effects. The adverse event of apheresis was considered as severe if it was life-threatening or leads to irreversible consequence with organ failure.

#### *Cell quantifications techniques*

The TNC, mononuclear cell (MNC), Plt and red blood cell (RBC) numbers in the patients' PB and/or AP samples were quantified using Advia-2120 blood counter (Bayer, Germany). The following monoclonal antibodies (mAbs) were used for the flow cytometric determination of CD45, CD34 and CD90 antigens/markers: anti-CD45-ECD, Immunotech, France; anti-CD34-FITC (class III antibody), BD Pharmingen, USA; anti-CD90-PE, Miltenyi Biotec GmbH, Germany). The samples were analyzed on FC-500 flow cytometer (Beckman-Coulter, FL, USA).

The SCs<sup>ish</sup> were determined as a constitutional part of CD34<sup>+</sup> cells in the „stem cell-region“ of the ISHAGE sequential gating protocol<sup>5–9</sup>. In our study, the original ISHAGE protocol was modified by introduction of anti-CD90-PE mAb into the analysis of CD90 expression on SC<sup>ish</sup>. Briefly, the SC<sup>ish</sup> were first gated on CD45 vs Side Scatter dot plot in order to separate the CD45<sup>+</sup> WBC from RBCs, Plts and other debris. From the primary gate on the CD45<sup>+</sup> events, the CD34<sup>+</sup> cells were identified on the CD34 vs Side Scatter dot plot by their expression of CD34 and characteristic light scatter properties. From the second gate on the CD34<sup>+</sup> events, the SC<sup>ish</sup> were back-gated on CD45 vs Side Scatter dot plot in order to separate “true” CD34<sup>+</sup> or SC<sup>ish</sup> with low CD45 fluorescence and low side scatter, from nonspecifically stained events – lymphocytes (CD45<sup>high</sup>), monocytes (CD45<sup>high</sup>) and higher Side Scatter) and granulocytes (high Side Scatter). In the next step, on the

Forward Scatter vs. Side Scatter dot plot, gated on events with low CD45 expression and low side scatter, the SC<sup>ish</sup> were identified by their size slightly larger than small lymphocytes and uniformly low side scatter. Finally, on the CD90 vs. Side Scatter dot plot, the selected „true“ CD34<sup>+</sup> (SC<sup>ish</sup>) cells were analyzed for CD90 expression. Cell viability was estimated on the basis of the 7-aminoaktinomycin D (7-AAD) flow cytometric assay (Immunotech, France)<sup>5,7,9</sup>.

The results obtained in this study were presented as a percentage of the SC<sup>ish</sup> per TNC count, absolute SC<sup>ish</sup> count in µL of the PB and AP, percentage of the CD90<sup>+</sup>SC<sup>ish</sup> expressed in SC<sup>ish</sup> and absolute CD90<sup>+</sup>SC<sup>ish</sup> count in µL of PB and AP (calculated on the basis of CD90<sup>+</sup>SC<sup>ish</sup> percentage).

For autologous SC-transplants, cryopreservation was performed according to our original five-step controlled-rate freezing protocol (with compensation of the released fusion heat), using dimethyl sulfoxide (DMSO; 10% final concentration) by Planer 560-16 equipment (Planer Products Ltd, UK), as it was earlier described<sup>13–15</sup>.

#### *Statistical analysis*

Descriptive data of the SC investigations were expressed as the mean value  $\pm$  standard error of mean (SEM) for each of the parameters examined. Statistical analyses were performed using GraphPad Prism 5 software. Differences were considered as statistically significant if  $p$  value was less than 0.05.

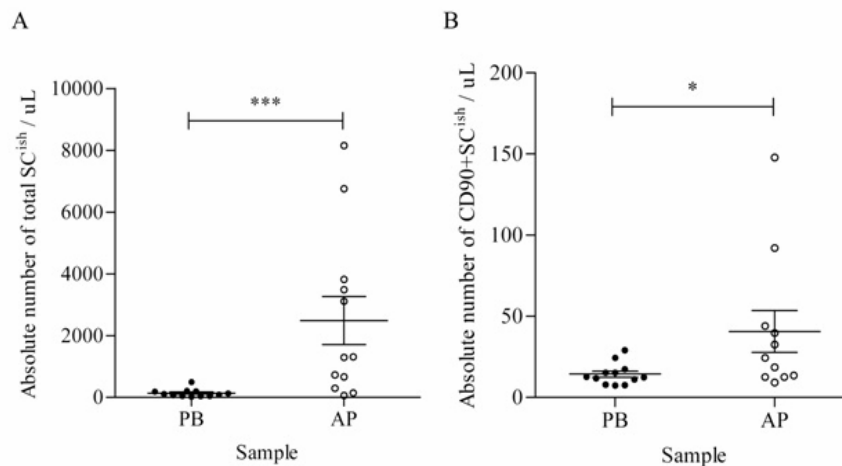
## **Results**

#### *Enrichment of total SC<sup>ish</sup> (absolute count) and the CD90<sup>+</sup>SC<sup>ish</sup> (relative frequency) cells in the apheresis product*

In this study, 12 patients subjected to autologous SC-transplant within the treatment of multiple myeloma were included. We initially assessed the quality of AP by comparing the absolute counts of targeted cells in the PB samples after mobilization with their yield in the AP. As expected, higher absolute count of total SC<sup>ish</sup> in the AP compared with the PB samples with very high statistical significance ( $2487 \pm 2678$  vs  $137.6 \pm 129.7$ ;  $p = 0.0007$ ; Figure 1A) was found. Similarly, the absolute count of CD90<sup>+</sup>SC<sup>ish</sup> was also significantly higher in the AP ( $40.7 \pm 42.8$  vs  $14.4 \pm 6.7$ ;  $p = 0.0266$ ; Figure 1B).

#### *The collection efficiency for the CD90<sup>+</sup>SC<sup>ish</sup> is lower than for the CD90<sup>+</sup>SC<sup>ish</sup> cells*

Next, we assessed the harvesting efficiency of CD90<sup>+</sup>SC<sup>ish</sup> by comparing of the CD90<sup>+</sup>SC<sup>ish</sup> yield with the total SC<sup>ish</sup> yield in the AP. With that purpose, we calculated the CD90<sup>+</sup>SC<sup>ish</sup> / total SC<sup>ish</sup> index by dividing the absolute count of CD90<sup>+</sup>SC<sup>ish</sup> with the absolute count of total SC<sup>ish</sup> and multiplied with 100, for both, the PB samples, as well as for the AP samples.



**Fig. 1 – Comparison of (A) total SC<sup>ish</sup> and (B) CD90<sup>+</sup>SC<sup>ish</sup> absolute counts between peripheral blood (PB) and apheresis product (AP) samples showing significantly higher counts in the AP samples (\* $p < 0.05$ ; \*\*\* $p < 0.001$ , respectively). (mean  $\pm$  SEM); Mann Whitney test). SEM – standard error of the mean.**

We found that, despite considerable positive correlation between these two indexes (Spearman  $r = 0.6503$ ;  $p = 0.0221$ ; Figure 2A), the CD90<sup>+</sup>SC<sup>ish</sup>/total SC<sup>ish</sup> indexes from the PB samples were significantly higher compared to the indexes from the AP samples ( $18.4 \pm 12.9$  vs  $9.3 \pm 11.7$ ;  $p = 0.0392$ ; Figure 2B). This finding indicates much higher CD90<sup>+</sup>SC<sup>ish</sup>/total SC<sup>ish</sup> proportion in the PB samples.

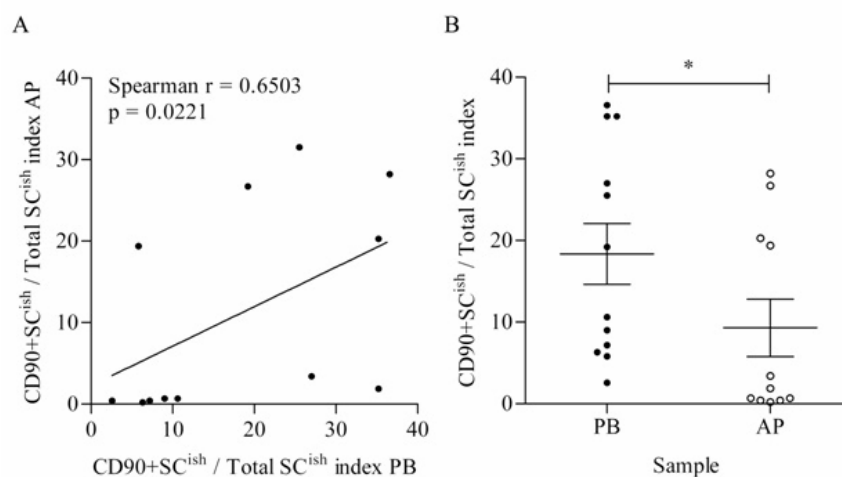
*The relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> correlates inversely with total SC<sup>ish</sup> in mobilized peripheral blood and apheresis product*

The relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> showed highly significant inverse relationship with the absolute count of total SC<sup>ish</sup> in both, the PB (Spearman's  $r = -0.8652$ ;  $p = 0.0003$ ; Figure 3A), and in the AP samples (Spearman  $r = -0.8140$ ;  $p = 0.0013$ ; Figure 3B).

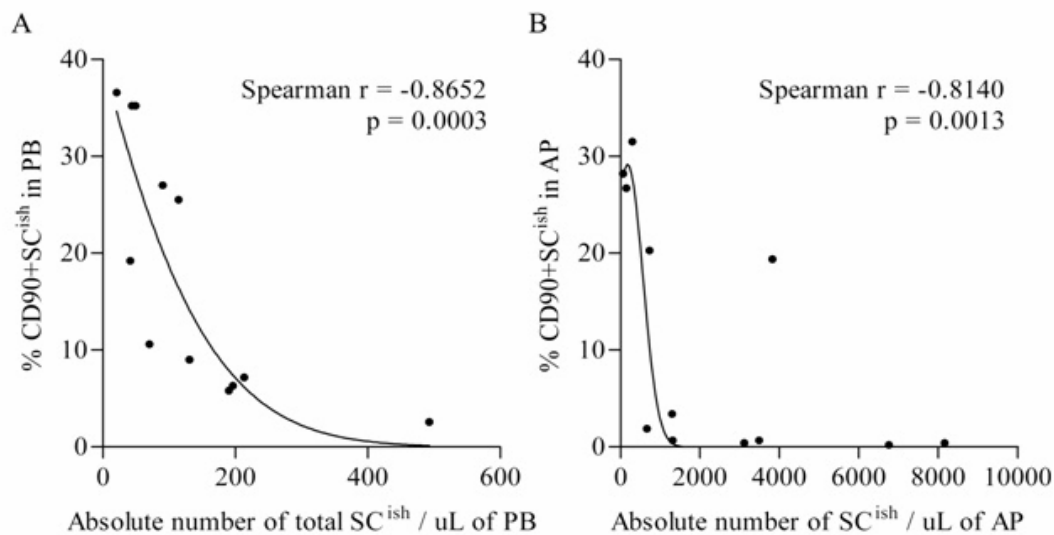
Interestingly, the relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> from the PB samples also showed highly significant inverse relationship with the absolute counts of total SC<sup>ish</sup> in the AP samples (Spearman's  $r = -0.8722$ ;  $p = 0.0002$ ; Figure 4A). In addition, we found that the patients with less than 10% of relative frequency of the CD90<sup>+</sup>SC<sup>ish</sup> in PB, had significantly higher total SC<sup>ish</sup> [since the higher appearance of mature SC<sup>ish</sup> (CD90<sup>+</sup>SC<sup>ish</sup>) subset] in the AP when compared with the patients with more than 10% of CD90<sup>+</sup>SC<sup>ish</sup> in their PB samples ( $5067 \pm 2249$  vs  $644 \pm 513.1$ ;  $p = 0.0025$ ; Figure 4B).

## Discussion

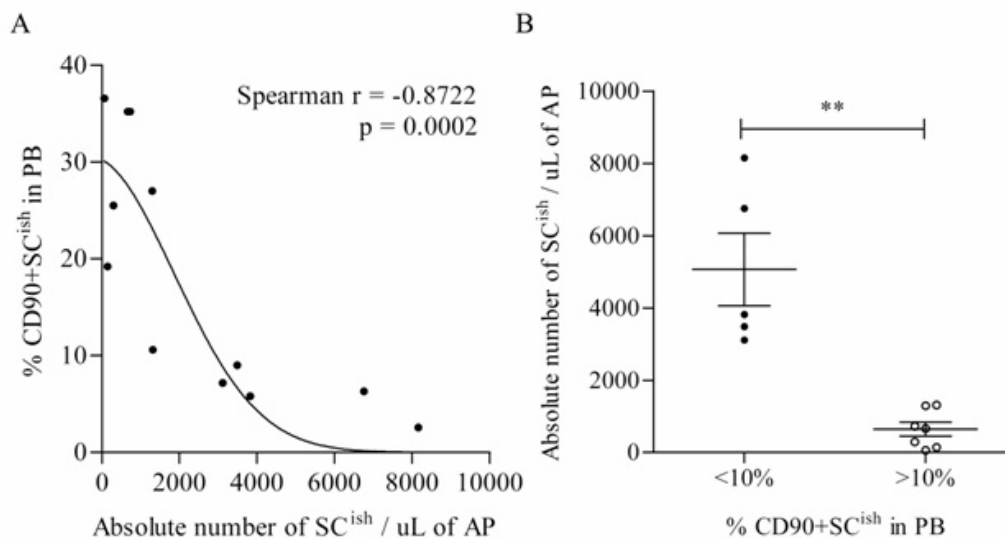
Although significantly progress in conventional medications has improved the prognosis of hematological malignancies, SC-transplant remains the most effective approach in obtaining disease free long-term survival of patients.



**Fig. 2 – (A) Correlation of peripheral blood (PB) and apheresis product (AP) CD90<sup>+</sup>SC<sup>ish</sup>/total SC<sup>ish</sup> indexes showing significant positive relationship (Spearman's correlation test); (B) Comparison between PB and AP CD90<sup>+</sup>SC<sup>ish</sup>/total SC<sup>ish</sup> indexes showing significantly higher values in the PB samples (\* $p < 0.05$ ; mean  $\pm$  SEM; Mann Whitney test). SEM – standard error of the mean.**



**Fig. 3 – Correlation between relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> and absolute counts of total SC<sup>ish</sup> in (A) peripheral blood (PB) and (B) apheresis product (AP) samples showing significant inverse relationship (Spearman's correlation test).**



**Fig. 4 – (A) Correlation between relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> and absolute counts of total SC<sup>ish</sup> in apheresis product (AP) samples showing significant inverse relationship (Spearman's correlation test). (B) Comparison of the SC<sup>ish</sup> absolute counts in the AP samples between the patients with less than 10% relative frequency of the CD90<sup>+</sup>SC<sup>ish</sup> and patients with more than 10% of the CD90<sup>+</sup>SC<sup>ish</sup> in their peripheral blood (PB) samples, showing significantly higher SC<sup>ish</sup> absolute counts in the group of patients with less than 10% of CD90<sup>+</sup>SC<sup>ish</sup> in PB (\*\* $p < 0.01$ ; mean  $\pm$  SEM; Mann Whitney test). SEM – standard error of the mean.**

However, high-dose treatment incorporates risks of conditioning-regimen related morbidity/mortality; accordingly it is limited for relatively younger patients in better clinical condition. This age limitation is regrettable since SC-transplant candidates in multiple myeloma are frequently older than 60 or even 65 years<sup>1</sup>.

The earliest SC collections from PB were accomplished in "steady state hematopoiesis" – by numerous procedures since late 1970s, and following cryopreservation was needed<sup>1,2,16</sup>. The cell harvesting is the same for allogeneic donors as for autologous patients. Vascular access, as mentio-

ned, is regularly realized using central/venous (jugular, subclavian or femoral) catheter.

In the course of cell harvesting, selection of the best collection system and determination of optimal timing for apheresis, are the most critical events. The most recent SC software design incorporates the Intermediate density layer (IDL) system using Spectra-Optia device<sup>1</sup>.

There are several advantages of PB as a source of the SCs as compared to the BM: absence of general anesthesia and multiple bone aspirations, higher CD34<sup>+</sup> yield in the AP and earlier hematopoietic reconstitution, as well as shortened

hospital stays and reduced transplant related morbidity. Commonly, the SC-engraftment is defined as the first of three days with neutrophil count greater than  $0.5 \times 10^9/L$  and Plt count exceeding  $20 \times 10^9/L$  (without transfusion support for 7 consecutive days)<sup>17</sup>.

Due to the reasons mentioned above, the number of patients treated by PB derived allogeneic, especially autologous SC-transplants is increasing worldwide<sup>1,2</sup>. Nowadays, the PB derived SCs are used for approximately 80% of allogeneic and practically for all autologous SC-transplants<sup>1,4</sup>.

Enumerated CD34<sup>+</sup> cell dose by flow cytometry is routinely performed to optimize timing of the PB stem cell collections and assess engraftment potential of the AP. Moreover, the immature CD34<sup>+</sup>/CD90<sup>+</sup> cells or PHPs have a capacity to initiate long-term hematopoiesis *ex vivo*, and according to some data, they are mobilized into blood to a maximum level a few days earlier than the peak mobilization of the total CD34<sup>+</sup> cells<sup>18</sup>. As mentioned, the CD34<sup>+</sup>/CD90<sup>+</sup> cells in the AP were a better predictor of Plt recovery than the total dose of the CD34<sup>+</sup> cells, and  $80 \times 10^4/kg$  of the CD34<sup>+</sup>/CD90<sup>+</sup> cells is the minimal essential dose capable of durable long-term engraftment<sup>11</sup>. Therefore, the measurement of the CD34<sup>+</sup>/CD90<sup>+</sup> cells is helpful for the evaluation of the grafts quality in the PB autologous SC-transplant.

There is an increasing interest in evaluation of the responsibility of the CD34<sup>+</sup> subsets for complete and long-term repopulation, as a marker of cell harvest quality<sup>1,10-12</sup>. In our initial clinical study, as a possible and potentially more objective collection predictor, the CD34<sup>+</sup>/CD90<sup>+</sup> subset evaluation was also recommended<sup>19</sup>. Precisely, on the basis of examination of the immature SC antigens and 7-amino-actinomycin D (7-AAD) viability, we suggested that the CD34<sup>+</sup>/CD33<sup>-</sup>, CD34<sup>+</sup>/CD38<sup>-</sup>, CD34<sup>+</sup>/DR<sup>-</sup>, CD34<sup>+</sup>/CD90<sup>+</sup> cells could be superior predictive factor over circulating the total CD34<sup>+</sup> count for an optimized collection-timing and outcome of autologous SC-transplant<sup>19</sup>. Other authors also confirmed that engraftment and hematopoietic recovery are not necessarily associated with cell dose of the total CD34<sup>+</sup> cells<sup>10</sup>. Thus, the CD34<sup>+</sup>/CD90<sup>+</sup> cells could be a useful quality marker for the AP<sup>10,19</sup>, especially for prediction of the Plt recovery (reduced hazard of the prolonged thrombocytopenia)<sup>11</sup>. Consequently, the CD34<sup>+</sup>/CD90<sup>+</sup> cells could be a practical predictive factor for complete and durable hematopoietic reconstitution<sup>10,11</sup>. The quantity of the mature CD34<sup>+</sup> subsets in the AP correlates obviously with engraftment rapidity<sup>1,2</sup>. However, data concerning the potential of the immature CD34<sup>+</sup> subsets (such as CD90<sup>+</sup>SC<sup>ish</sup>) for long-term marrow repopulation are still not completely clarified.

Our previous preclinical research (using standardized cell harvesting protocols) confirmed that Spectra-Optia resulted with superior collection efficiency compared to Cobe-Spectra for the CD34<sup>+</sup> cells (CE2[%]<sub>CD34<sup>+</sup></sub>)<sup>20</sup>. The CE2[%]<sub>CD34<sup>+</sup></sub> was calculated on the basis of the CD34<sup>+</sup> count in the AP versus their number in processed whole blood<sup>20</sup>. We estimated that the "predictive-value" of circulating total CD34<sup>+</sup> cells for the SC harvesting could be enhanced or improved by determination of the relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> in patients' PB.

In this study the relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> demonstrated inverse correlation with the absolute count of total SC<sup>ish</sup> in both, the PB and AP. Accordingly, on the basis of the inverse correlation between the PB and the AP counts of CD90<sup>+</sup>SC<sup>ish</sup> observed in present study, it is possible that the level of the collection efficiency of both existing (Cobe-Spectra and Spectra-Optia) apheresis systems is inferior for the immature (CE[%]<sub>CD90+SC<sup>ish</sup></sub>) as compared to the mature (CE2[%]<sub>CD34<sup>+</sup></sub>) cells from mobilized PB. However, we believe that the lower CD90<sup>+</sup>SC<sup>ish</sup> yield is not a consequence of inferior CE[%]<sub>CD90+SC<sup>ish</sup></sub>, but possibly a result of several still not fully resolved immature SC features, such as cytomorphological and biophysical (intracellular granulation, cell-density, etc) parameters. Using the „dye-efflux“ method for SC-sorting, Radley et al.<sup>21</sup> defined more precisely ultrastructural characteristics of the most primitive SCs in murine BM and provided a basis for studying the structural changes that occur with progressive activation and differentiation of immature hematopoietic SCs (HSCs) toward their progeny of lesser proliferative capacity. The structural changes could likely have effect on the density and consequently "centrifugation-properties" of these cells. Sharma et al.<sup>22</sup> showed that the CD90<sup>+</sup> cells were predominantly found within the subpopulation of the SCs with low CD34 and very low CD45 antigen expression, in the patients with hematological malignancies. They also showed that those CD90<sup>+</sup>CD34<sup>dim</sup>CD45<sup>very dim</sup> cells were the smallest among all other examined subpopulations (including CD133<sup>+</sup> and CD117<sup>+</sup> CSs with the different CD45 antigen expression) with an electronic volume of 87.1–143.7  $\mu m^3$  corresponding to a diameter of 5.5–6.5  $\mu m$ . In the review article, Kucia et al.<sup>23</sup> postulate that the fraction of the SCs, small in their size, could be easily lost during collection/isolation protocols based on gradient or centrifugation. Our finding of significantly lower enrichment index for CD90<sup>+</sup>SC<sup>ish</sup>, compared with enrichment index of the total SC<sup>ish</sup>, is in accordance with the aforementioned earlier investigations.

In this study an intriguing observation is the inverse relationship between relative frequencies of the CD90<sup>+</sup>SC<sup>ish</sup> and absolute counts of the total SC<sup>ish</sup> in PB, suggesting that continual increase in absolute counts of the total SC<sup>ish</sup>, during the G-CSF-induced mobilization, is not followed by an appropriate increase in the CD90<sup>+</sup> cells. The G-CSF acting – either as an endogenously produced after chemotherapy or as exogenous or administered factor (rHuG-CSF), or a combination of both – is considered to be an initial signal for HSC mobilization, explained by several proposed pathways<sup>24</sup>. Nevertheless, there are data describing the superior effects of some new agents, such as mozobil or plerixafor (which is an antagonist of the alpha chemokine receptor CXCR4) in mobilizing the immature HSC, capable for long-term repopulation (LT-HSC)<sup>25</sup>.

## Conclusion

We speculate that the inferior CD90<sup>+</sup>SC<sup>ish</sup> yield in the AP is not a consequence of the inferior collection efficacy – but most likely a result of several still not fully understood

od/clarified immature SC cytomorphological and/or biophysical features. Thus, when the chemotherapy/G-CSF is used for mobilization, there are some logical questions to ask- whether we should follow the absolute count of the total SC<sup>ish</sup>, or, whether we should test for relative frequency of CD90<sup>+</sup>SC<sup>ish</sup> prior to apheresis.

To reach the final conclusions, it is essential to conduct further controlled and larger SC-investigations (our clinical study has been already initiated) concerning the correlation

between circulating and harvested SCs, and patients' hematopoietic recovery.

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