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Mobilization of Haematopoietic Stem Cells CD34+ in Patients undergoing Elective Lung Resection: Observational Pilot Study

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Abstract

Background: Blood haematopoietic stem cells expressing the CD34 cell surface marker (CD34+) may differentiate under appropriate stimula into a variety of cell types to repair damaged organs. Purposes of this study were to verify whether lung surgical trauma can activate bone marrow mobilizing peripheral CD34+ cells and the time course of their activation.

Methods and findings: In fifty-one patients undergoing elective surgical lung resection under general anesthesia, ASA II-III, without chronic obstructive pulmonary disease, blood samples for white blood cells (WBC) and circulating blood CD34+ were collected before surgery (T0), at 24 (T24h) and 72 (T72h) hours, 5 (T5d) and 7 (T7d) days postoperatively. CD34+ cells quantification was performed by flow cytometry. A hierarchical clustering analysis identified two clusters based on the CD34+ time course: in cluster #1 (14 patients), CD34+ decreased by 46% on T24h (p=0.0023 *vs* T0) and then increased constantly reaching 230% on T7d (p=0.00016 *vs* all previous time points), in cluster #2 (37 patients), CD34+ remained stable throughout the study. Patients entering cluster #1 were younger (58+4 years *vs* 66+1 years, p=0.03), had a higher baseline CD34+ count (on T0 CD34+ 2.55+1.5 n/10-3 *vs* 1.2+0.57 n/10-3, p=0.0023) and the pre-operative FEV1/FVC was significantly higher (95.66 *vs* 76.6, p=0.02).

Conclusions: Our data show that peripheral blood CD34+ are mobilized from the bone marrow in patients undergoing lung resection and their count seems to be correlated with WBC and CD34+ basal values and with patients age.

Keywords: Haematopoietic stem cells, Blood cells, Bone marrow cells

Introduction

Haematopoietic stem cells (HSC) are the precursors of all peripheral blood cells, and can differentiate into all hematological cellular lineages, being self-sufficient thanks to a pool of resting stem cells in the bone marrow that proliferate in response to neuro-humoral signals. The HSC surface membrane exhibits antigens that allow them to be identified as CD34+ [1,2]. In humans, the CD34+ cell surface marker has been shown to characterize cells with a haematopoietic activity which is present on approximately 0.5-3% of bone marrow and <1% of peripheral blood mononuclear cells [2].

CD34+ cells represent a heterogeneous fraction of enriched endothelial/hematopoietic progenitors in peripheral blood that differentiate into endothelial cells and mesenchymal cells [3-5]. The potential of human HSC to migrate into solid organs in response to stress signals in order to repair non-haematopoietic tissues such as myocardium and liver has been demonstrated in experimental and clinical studies [4,6,7]. Moreover, musculoskeletal trauma may cause a systemic increase of circulating CD34+ haematopoietic progenitor cells through bone marrow cells expansion and subsequent release into circulation [8].

As regards lung injury, a study in mice demonstrated that HSC contribute to cell lineages from the lung alveolar epithelium and that lung injury alone is sufficient to promote circulating stem cell migration [9]. However, few data are available on the time course of HSC migration following lung surgical trauma.

Purpose of this study was thus to test if lung surgical trauma in patients undergoing elective lung resection evokes an increase of HSC, and the time course. The secondary aim was to evaluate factors predictive of HSC variations over time in such patients.

Materials and Methods

Patients and protocol

After obtaining approval from the O.O.R.R. hospital ethics committee of Foggia (n.66, 03/02/2012) and written informed consent from each patient, the study was performed in patients scheduled consecutively to undergo elective lung parenchymal resection. Inclusion criteria were age>18 years, ASA physical status II and III. Patients with clinical signs of systemic inflammation (white blood cells (WBC)>10,000 cells/µl), and/or preexisting lung or cardiac disease, or pathologic lung function were excluded from the study.

Pre-operative spirometry was carried out in all patients undergoing elective lung parenchymal resection for evaluation of forced expiratory volume in 1 second-forced vital capacity ratio (FEV1/FVC).

Upon their arrival in the operating room, patients were pre-medicated with midazolam 0.03-0.04 mg/kg, standard monitoring devices [electrocardiogram and pulse oximeter (Intellivue MP40 monitor, Philips, Boeblingen, Germany)] were applied and the radial artery was cannulated (Radial Artery Catheterization Set, Arrow International, Reading, PA) for continuous monitoring of blood pressure. Normal saline was administered to patients intravenously at 8ml/kg before the anaesthesia induction and at 5 ml/kg/h during the maintenance of anaesthesia. Propofol 2 mg/kg, fentanyl 3 mcg/kg and succinylcholine 1 mg/kg were used for anaesthesia induction. Afterward the trachea and the left bronchus were intubated with a left double lumen endotracheal tube of appropriate size (Rushelit Rush AG Lab, Waibling, Germany). Correct double lumen endotracheal tube position was checked by fiber optic bronchoscopy and confirmed after positioning patients in lateral decubitus. At one lung ventilation (OLV) time, the lumen of the non ventilated lung was left open to the atmosphere.

Anaesthesia was maintained with an infusion of propofol 5 mg/kg/h, remifentanil 0.1-0.2 mcg/kg/min and cisatracurium 1.5 mcg/kg/min and bi-spectral index (BIS) (Aspect A-2000 ®; Aspect Medical System, Newton, MA) was used for monitoring anaesthesia depth and for modulating the infusion rate of propofol to maintain a BIS value between 40 and 60. The patients were ventilated through a Servo Ventilator 900C (Siemens-Elema AB, Berlin, Germany) with a square flow waveform with a tidal volume (Vt) of 8 ml/kg, respiratory rate of 12 breath/min, inspiratory time of 33% and an inspiratory pause of 20%. The inspiratory oxygen fraction was set at 50% and increased as needed to maintain the SaO2 ≥95%. During OLV, Vt was reduced to 6 ml/Kg, while the other parameters were unvaried.

Blood heparinized venous samples for WBC count, circulating haematopoietic cells and blood count were recorded before surgery (T_0) and at four time points postoperatively: 24 (T_{24h}) and 72 (T_{72h}) hours and 5 (T_{5d}) and 7 (T_{7d}) days after surgery.

Postoperative complications such as fever > 38°C, systemic infection (defined as WBC rate>10,000 cells/µl) were recorded.

CD34+ isolation

CD34+ cells were detected by a modified ISHAGE (International Society of Hematotherapy and Graft Engineering) method [10]. In the present study, we modified the basic ISHAGE method based on a four-parameter flow cytometry (CD45 FITC/CD34 PE staining, side and forward angle light scatter) that allow to generate an absolute CD34+ count by incorporating the leukocyte count from an automated analyser (two-platform method), with the addition of a known number of Flow-Count fluorospheres. To reduce errors inherent to sample washing/centrifugation, we implemented ammonium chloride lysis, no-wash no-fix sample processing. These modifications convert the basic protocol into a single-platform method to determine the absolute CD34+ count directly from a flow cytometer, and form the basis of the Stem-Kit (Coulter/Immunotech.). Quantification of CD34+ cells was performed by flow Cytometry (COULTER EPICS XL-MCL, Instrumentation Laboratory). Briefly, peripheral blood samples were collected aseptically into sterile evacuated blood collection tubes with anticoagulant EDTA, and stored at room temperature (18-25°C) until staining within 24 hours. Prior to sample preparation, blood cells (WBC) were counted. If the WBC rate was >30 × 10⁹ WBC cells/L, the sample was discarded. Specific cell surface staining was accomplished by incubating duplicate samples of a biological specimen with the two colour CD45-FITC/CD34-PE reagent. An additional test of the same sample is stained with the CD45-FITC/ IsoClonic™ Control-PE reagent to check the non-specific binding of the CD34-PE monoclonal antibody. 7-AAD Viability Dye, a nucleic acid dye that binds to accessible base pairs, is used to distinguish between viable and nonviable cells. Each specimen sample was divided into three parts: 45/34/7-AAD *1, 45/34/7AAD *2, 45/CTRL/7AAD.

In the first and second samples, 20 µL of CD45-FITC/CD34-PE Reagent were added; and 20 µL of CD45-FITC/IsoClonic Control-PE Reagent was pipetted into the 45/CTRL/7AAD tube. In all tubes 20 µL of 7-AAD Viability Dye were pipetted. Using a positive displacement pipette, 100 µL of the patient sample were pipetted into the tubes that were immediately vortexed for 5 seconds

and incubated at room temperature (18-25°C) for 20 minutes, protected from light. After incubation, 2 ml of prepared $1 \times NH_4Cl$ Lysing Solution were added to each tube, which was vortexed immediately for 5 seconds. The tubes were then incubated at room temperature for 10 minutes, protected from light. Prior to acquisition by Cytometry COULTER EPICS XL-MCL, 100 µL of Stem-Count Fluorospheres were added into each of the three tubes, and the tubes were immediately vortexed for 5 seconds. Each fluorosphere contained a fluorochrome with a length of fluorescence ranging between 525 to 700 nm when excited at 488 nm [7].

Statistical analysis

The sample size calculation was performed based on a study published by Laing et al. [11], which described the number of CD34+ cells in patients with isolated, low-energy, closed tibial fractures. A total of 16 patients (8 patients per group) were needed to obtain a desired statistical power of 0.9 (anticipated effect size (Cohen's d):2.9; probability level for a two-tailed hypothesis (p):0.001). Data are reported as mean ± standard deviation (SD) or 95% confidence limits (CL) as appropriate. Cluster analysis was used to categorize patients:firstly a hierarchical agglomerative clustering [12] was performed by complete-linkage clustering to evaluate how many "natural" clusters that could be labelled in a meaningful manner were formed by our patients; afterwards the *k*-mean clustering method [13] was applied on the basis of this number of clusters so as to assign observations to each cluster. Cluster analysis was conducted using the CD34+ count recorded at T_0, T_{24h}, T_{72h}, T_{5d} and T_{7d}. Subsequently, intra- and intergroup comparison between the two clusters in terms of CD34+, monocytes and WBC count over time was performed by means of repeated measures two-ways analysis of variance (ANOVA). Finally, one-way ANOVA or Chi-square test as appropriate were used to evaluate whether clusters were significantly different in terms of baseline characteristics (age, sex, FEV_1/FVC, ASA score, duration of surgery, postoperative presence of fever), in order to identify factors predictive of haematopoietic stem cells variations over time in such patients. The test of Tukey was used for post hoc analysis. A p value <0.05 was considered significant.

Statistics was performed using Statsoft.Inc (2011) STATISTCA (data analysis software system, version 10, www.statsoft.com, Padova, Italy).

Results

75 patients undergoing elective surgical lung resection were considered for possible inclusion. We excluded 20 patients because they did not meet inclusion criteria. Consequently, 55 participants were recruited to the study and underwent CD34+ sampling: 2 patients were lost to follow-up due to their transfer to another hospital, in 2 patients data were missing. Therefore, the final statistical analysis was performed on 51 patients. The enrolment flow diagram is reported in **Figure 1**.

The hierarchical clustering analysis identified two clusters based on the identification of the CD34+ count time course and the k-mean cluster analysis allowed us to identify members of each cluster for the subsequent ANOVA analysis, that confirmed a significant difference in the mean CD34+ time course between the two clusters **(Figures 2 and 3)** : in cluster #1 (14 patients), CD34+ decreased by 46% on T_{24h} after surgery (p=0.0023 *vs* T_0) and then increased constantly, reaching 230% on T_{7d} (p=0.00016 *vs* all the other time points); in cluster 2 (37 patients), CD34+ remained substantially stable throughout the study. Intragroup comparison showed that patients in cluster 1 had higher baseline values of CD34+ (p=0.0023 *vs* cluster 2 on T_0). The two clusters were different in terms of demographic and baseline blood cells count: patients in cluster 1 were younger (58 ± 4 years *vs* 66 ± 1 years in cluster 2, p=0.03), and had higher T_0 baseline values of WBC, and CD34+/monocytes count (p=0.00016 respectively (**Table 1**).

Pre-operative FEV_1/FVC was significantly higher in cluster 1 (95.7 *vs* 76.6, p=0.02).

The mean WBC time course did not vary statistically in the two clusters. Circulating CD34+cells were more numerous in cluster 1 patients at every time points but T_{24h}. No differences regarding the remaining demographic parameters were found between the two clusters.

Discussion

The main results of the present study are a) peripheral blood CD34+ are mobilized from the bone marrow in patients undergoing lung resection; b) a basal value of CD34+> 2 cells/ml, together with patients age, seem to be predictive factors for CD34+ stem cells mobilization; c) the CD34+ time course was characterized by a sharp consumption of circulating cells in the first 24 h after lung resection, followed by a net increase from 72 h until 7 days after the parenchymal injury.

Before discussing our results, some preliminary considerations on stem cells physiopathology are required. Up to now, it has been recognized that HSC can home to and/or participate in repairing of missing or damaged lung tissue [13-15]. The HSC compartment is phenotypically heterogeneous: many different surface markers have been studied to identify HSC from mouse bone marrow, but a consensus as to which markers are consistently expressed on these cells remain unclear, since there are populations of HSC that are positive and negative for CD34 expression [16-20]. It may be that the expression of CD34 is reversible and related to cell cycle activation [21].

Circulating CD34+ progenitor cells gained importance in the field of regenerative medicine due to their potential to home in injury sites and differentiate into cells of endothelial and osteogenic lineages [22-27].

Our data show a significant increase of peripheral blood CD34+ cells in patients undergoing lung resection. Based on our results we speculate that the differences in postoperative CD34+ cells release could be related to patients' bone marrow 'functional reserve': indeed, patients showing, after a reduction at T24h, a progressive increase in circulating CD34+ cells had a number of circulating CD34+ cells significantly higher at baseline. Very likely, this can be explained by the groups different age distribution, younger patients without respiratory function impairment belonging to cluster 1: mounting evidence supports the idea that in tissue-specific stem cell compartments, aging is invariably
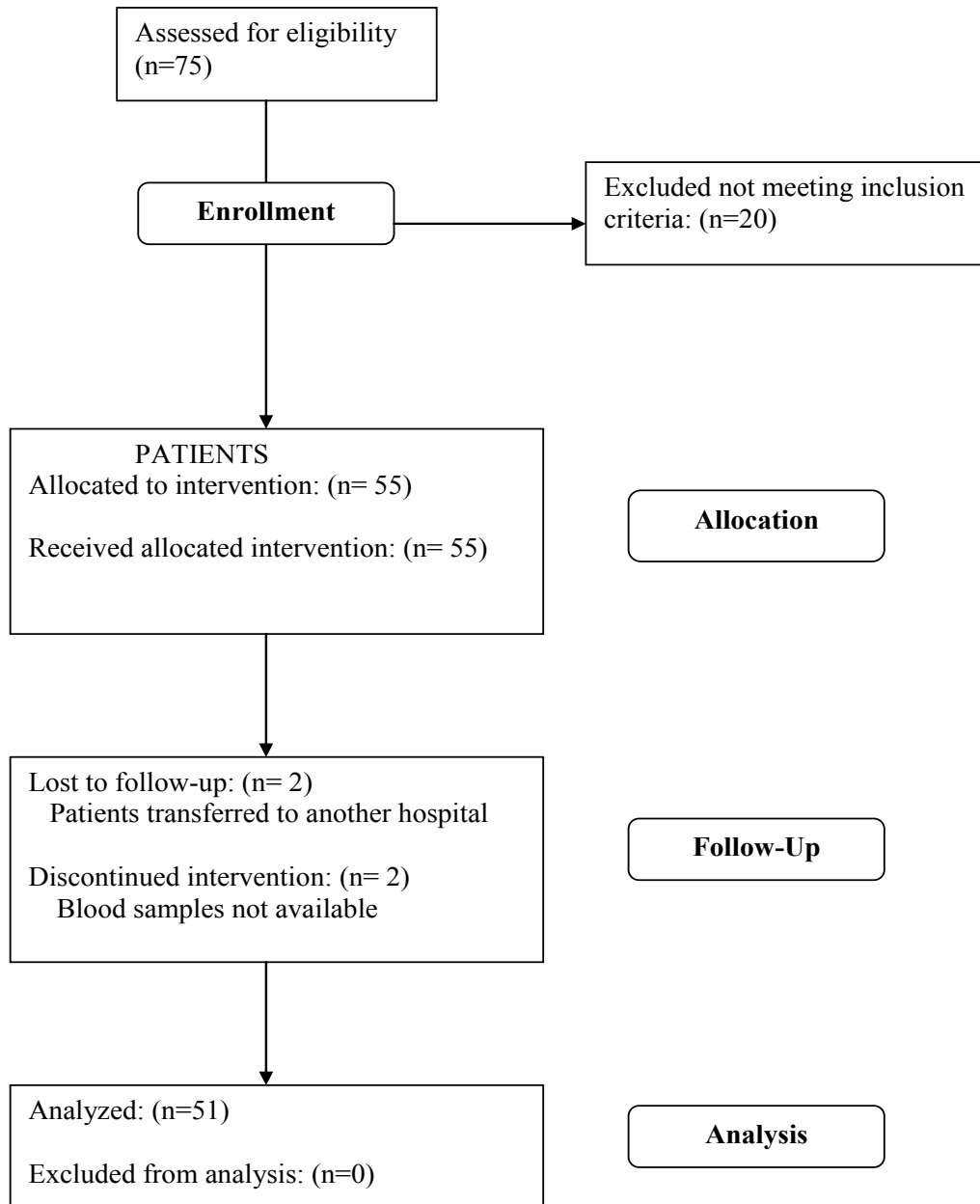
Figure 1 Flow-diagram of the different trial phases.

accompanied by a reduced capacity to adequately maintain normal homeostatic tissue maintenance and regenerative response after injury [24,28]. In recent years, many theories have been proposed to justify the stem cell decline with aging, related to cell proliferation, differentiation, cell cycle phases, alterations in gene expression and cytokine and growth factor production [13,29]. The observation of a reduced cellularity of bone marrow in the elderly, including CD34+ bone marrow cells support the hypothesis of the human haematopoietic system decrease in advancing age [30,31].

Furthermore, Fadini et al. postulated an age-dependent CD34+ exhaustion between patients and control and demostrated that circulating CD34+ decrease is related to disease severity, especially in patients with restrictive lung disease *vs* chronic obstructive

pulmonary disease (COPD) or controls (respectively FEV1/FVC ratio 78.59 *vs* 48.31 or 71). The oxigen therapy, reflecting severity of hypoxia in COPD, could lead a compensatory effect of hypoxia associated with higher number endothelial progenitor cells mobilization in COPD [32].
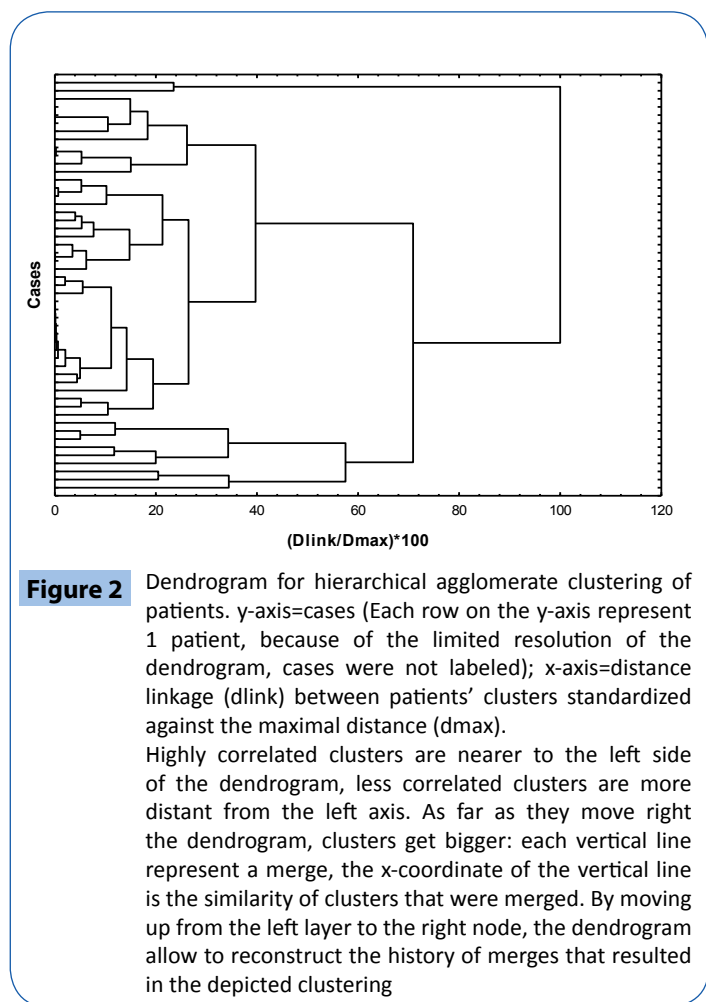
In our study the baseline FEV1/FVC ratio was statistically lower in cluster 2 (76.6 *vs* 95.66 in cluster 1, p=0.02) although the patients were free from clinical and instrumental evidence of chronic lung disease. We postulate that the decreased FEV1/FVC ratio is age-related.

Finally, we observed higher baseline values of WBC in the cluster of patients releasing CD34+ cells in the postoperative period. Interestingly, associations among WBC and CD34+ cells

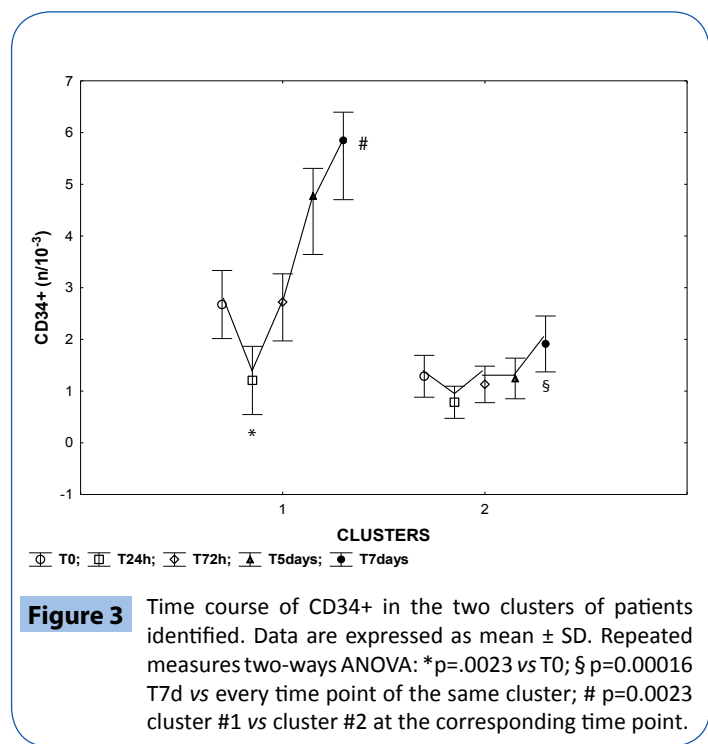**Table 1** Baseline characteristics of the two clusters of patients.

	Cluster 1 (N 14)	Cluster 2 (N 37)	
Age (years)	58 ± 4.2	66 ± 1.5	p<0.003
Sex (M:F)	4-Oct	28/9	NS
FEV1/FVC	95.7	76.6	p<0.02
Surgery duration (min)	167 ± 23	183 ± 13	NS
ASA score (II:III)	8-Jun	19/18	NS
Fever N (%)	8 (55%)	12 (32%)	NS
White Blood Cells (cells/μl)	7327 ± 867	5864 ± 269	P<0.03
Monocytes (cells/μl)	569 ± 85	477 ± 36	NS
CD34$^+$ (cells/μl)	2.6 ± 0.39	1.20 ± 0.09	p<0.0001
CD34$^+$/Mono	0.203 ± 0.033	0.098 ± 0.078	p<0.0001

ASA=American Society of Anesthesiologists physical status; CD34+=haematopoietic cells exhibiting the CD34 cell surface marker; FEV1/FVC=forced expiratory volume in 1 sec to forced vital capacity ratio.

Figure 2 Dendrogram for hierarchical agglomerate clustering of patients. y-axis=cases (Each row on the y-axis represent 1 patient, because of the limited resolution of the dendrogram, cases were not labeled); x-axis=distance linkage (dlink) between patients' clusters standardized against the maximal distance (dmax).

Highly correlated clusters are nearer to the left side of the dendrogram, less correlated clusters are more distant from the left axis. As far as they move right the dendrogram, clusters get bigger: each vertical line represent a merge, the x-coordinate of the vertical line is the similarity of clusters that were merged. By moving up from the left layer to the right node, the dendrogram allow to reconstruct the history of merges that resulted in the depicted clustering

Figure 3 Time course of CD34+ in the two clusters of patients identified. Data are expressed as mean ± SD. Repeated measures two-ways ANOVA: *p=.0023 vs T0; § p=0.00016 T7d vs every time point of the same cluster; # p=0.0023 cluster #1 vs cluster #2 at the corresponding time point.

in predicting the optimal timing of peripheral blood CD34$^+$ stem cell collections by apheresis have been described in literature, especially in the hematological field [33-35].

The present study has some limitations: a) firstly, the study was conducted in otherwise healthy patients undergoing elective surgery and none of them showed postoperative complications; more data are needed to study the CD34$^+$ time course in patients with co-existing cardiopulmonary diseases and/or postoperative complications; b) there was a certain lack of homogeneity in the surgical procedures among our patients, that must be taken into account when interpreting our data; c) we could not calculate postoperative FEV1/FVC ratio.

Conclusions

Our observational pilot study showed that peripheral blood CD34$^+$ are mobilized from the bone marrow in patients undergoing lung resection and their count seems to be correlated with WBC and CD34$^+$ basal values and with patients age. The data do not allow to attribute this phenomenon to the surgical resection per se or the anesthesia or the lung disease and future work will be required to obtain this information.

Advances in stem cell research have explored many therapeutic

avenues [36,37] and a better understanding of the mechanisms of mobilization may promote major future suggestion, and provide a means of direct and therefore more predictable, and higher-yield mobilization.

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