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Modulating factors of progenitor stem cell kinetics in STEMI patients undergoing primary angioplasty – a pilot study

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Abstract

Aims: To investigate the circulating progenitor stem cells (cPCs) count evolution during seven days hospitalization period in ST segment elevation myocardial infarction (STEMI) patients, and to correlate their evolution with some clinical and angiographic parameters. *Materials and Methods*: Twelve Caucasian patients with STEMI undergoing primary percutaneous coronary intervention (PCI) were enrolled. Blood samples were obtained in the emergency room and then daily, for seven days, we evaluated the number of cPCs ($CD34^+CD45^+$, $CD133^+CD34^+CD45^+$, $KDR^+CD34^+CD45^+$ and $KDR^+CD133^+CD34^+CD45^+$) by flow cytometry using fluorochrome-marked specific monoclonal antibodies. *Results*: There is a statistically significant increase in cPCs counts in the following days after STEMI, with a different behavior depending on their phenotype. Mature cPCs ($CD34^+CD45^{dim}$, $KDR^+CD34^+CD45^{dim}$) have two fairly similar peaks, first around the third day of evolution followed by a short decrease and a new raise in the seventh day, the more immature cPCs ($CD133^+CD34^+CD45^{dim}$, $KDR^+CD133^+CD34^+CD45^{dim}$) have just one spike on the third day, and then almost disappear from the peripheral circulation. In a multivariate regression analysis, preprocedural TIMI (Thrombolysis In Myocardial Infarction) flow, postprocedural myocardial blush and LVEF (Left Ventricular Ejection Fraction) proved to be independent predictors for cPCs variation in the first week after STEMI. *Conclusions*: In our study, we demonstrated that all four main phenotypes of circulating progenitor stem cells boosted up in the next days after STEMI, with different patterns depending on cell type; preprocedural TIMI flow, postprocedural myocardial blush and LVEF proved to be independent predictors for cPCs mobilization in the first days after STEMI.

Keywords: circulating progenitor stem cell, ST segment elevation myocardial infarction, percutaneous coronary intervention.

Introduction

Despite the remarkable progress in knowledge gained in the last years, cardiovascular disease (CVD) is still the leading cause of death in Europe, accounting for over 4.3 million deaths each year (48% of all deaths) [1]. Vascular endothelial dysfunction plays a major role in the pathogenesis of CVD. Although initially considered a passive inner wrap of the vessel wall, the endothelium has now been proved to be an active organ with vital functions, such as regulation of vascular tone and permeability, coagulation, inflammation and angiogenesis [2].

The permanent injury of the vascular endothelium leads to endothelial dysfunction, characterized by reduced nitric oxide (NO) production and progressive loss of endothelial cells (ECs). Although endothelial restoration was considered to be entirely mediated by the neighboring ECs, recent studies demonstrated the involvement of circulating endothelial progenitor cells (EPCs) in endothelial regeneration [3], administration or mobilization of EPCs improving the endothelial repair after focal denudation [4, 5].

Additional studies demonstrated that circulating EPCs that originate in the bone marrow are attracted to the site of injury (*e.g.*, ischemia) where they contribute both directly and indirectly to the growth of new blood vessels, thus having a major role in the preservation of endothelium integrity and function [6]. The critical discovery made by Asahara's group that postnatal vasculogenesis does really occur, offered novel opportunities to cardiac repair. After birth, neovascularization is not based entirely on angiogenesis (mature, differentiated endothelial cells sprouting from pre-existing blood vessels, migrate and proliferate to form new vessels), but also on vasculogenesis (spontaneous new blood-vessel formation from circulating or tissue-resident endothelial stem cells, which proliferate into de novo endothelial cells). From the time of their first mention more than 15 years ago [7], the definition of EPCs as co-expressing of CD34 and kinase insert domain receptor (KDR) has been challenged by further studies [8–12] which have revealed that the idiom 'EPC' does not label a single cell type, but rather depicts a variety of cells able to differentiate into the endothelial lineage.

Previous studies demonstrated that cardiovascular risk factors are associated with reduced levels of circulating EPCs [13, 14] and decreased levels of circulating EPCs independently predict atherosclerotic disease progression [15].

Since there is growing evidence that the level of circulating EPCs is influenced by the cardiovascular risk factors and by the endothelial injury (*e.g.*, myocardial ischemia, percutaneous coronary intervention), we aim to investigate the circulating progenitor stem cells (cPCs) count evolution during seven days hospitalization period in ST segment elevation myocardial infarction (STEMI) patients. The study was performed in the Department of Cardiology, Emergency Clinical Hospital of Bucharest, Romania.

Materials and Methods

Characteristics of study patients

Twelve Caucasian patients (nine men and three women, mean age 57.25±10.82 years) with acute ST segment elevation myocardial infarction undergoing primary percutaneous coronary intervention (PCI) were enrolled in this study after informed consent. Patients with concomitant inflammatory or malignant disease, alcohol or drug addiction were excluded.

Blood sampling, characterization and quantification of circulating progenitor cells

Blood samples were collected aseptically by venipuncture in sterile EDTA vacutainers; the initial sample was obtained in the emergency room (before the catheterization), whereas the others were taken daily, at the same time (7 a.m.), in a fasting state, during next seven days (common hospitalization period for STEMI patients). The average time from the onset of the thoracic pain until the first blood collection was 6.83±3.21 hours. EDTA collection tubes were stored at room temperature and processed within 2 to 10 hours, using a lyse-no wash protocol. In order to determine the absolute number of cPCs, we added a precise amount of 50 µL of whole blood by reverse pipetting in TruCOUNT Tubes (Becton, Dickinson & Co.). The blood was incubated for 15 minutes in the dark at room temperature with monoclonal antibodies against human CD45 (PerCP; Becton, Dickinson & Co.), anti-CD34 (FITC; Becton, Dickinson & Co.), anti-KDR (PE; R&D Systems) and anti-CD133 (APC; Miltenyi Biotech). Subsequently, the samples were lysed before flow cytometry analysis by adding 450 mL lysing buffer (1X Pharm Lyse; Becton, Dickinson & Co.) and incubated 30 minutes in the dark at room temperature. The cell suspension consisting of heterogeneous cell populations was evaluated shortly afterwards, using appropriate gating strategy on a FACSCalibur machine equipped with a BD CellQuest Pro software (Becton, Dickinson & Co.). The gating strategy applied for $CD34^{+}$ $CD45^{dim}$ cells (*i.e.*, true stem cells) enumeration was an adaptation of *Assessment of CD34+ Cell Count in Thawed Cord Blood Units Operative Protocol* [16]. This approach made isotype controls unnecessary since the gating strategy excluded cells that non-specifically bind to CD34. We measured the numbers of $CD34^{+}CD45^{dim}$, $CD133^{+}CD34^{+}$ $CD45^{dim}$, $KDR^{+}CD34^{+}CD45^{dim}$ and $KDR^{+}CD133^{+}CD34^{+}$ $CD45^{dim}$ cells in duplicate, and the mean value was used. Roughly 50 000 $CD45^{+}$ cells were acquired.

Briefly, after obtaining a CD45 *versus* side scatter (SSC) dot plot (Figure 1a), an initial region ($CD45^{+}$) was set to comprise all $CD45^{+}$ events including $CD45^{dim}$ and $CD45^{bright}$ and also the TruCOUNT beads, letting out the $CD45^{-}$ events (red blood cells, platelets and other debris) (Figure 1b). The 2nd region identified $CD34^{+}$ cells (Figure 1c). $CD45^{+}$ region also included two smaller regions: lymphs, which defines lymphocytes as $CD45^{bright}$ SSC^{low} cells, and dim $CD45^{+}$ respectively (Figure 1, b and d). An exclusion gate was located on a forward scatter (FSC) *versus* SSC dot plot in order to get rid of the residual debris (Figure 1e). Also, on FSC/SSC it was placed a region to contain cells with intermediate characteristics between lymphocytes and monocytes (R4) (Figure 1f). TruCOUNT beads region was depicted on a CD34/CD45 dot plot (Figure 1g). The real $CD34^{+}CD45^{dim}$ cells were branded after simultaneously satisfying the criteria of all four regions: $CD45^{+}$, $CD34^{+}$, dim $CD45^{+}$, R4, but not TruCOUNT beads or exclusion. The $CD34^{+}CD45^{dim}$ cells were then displayed on a KDR/CD133 dot plot in order to obtain the subsequent cPCs subpopulations (Figure 1h).

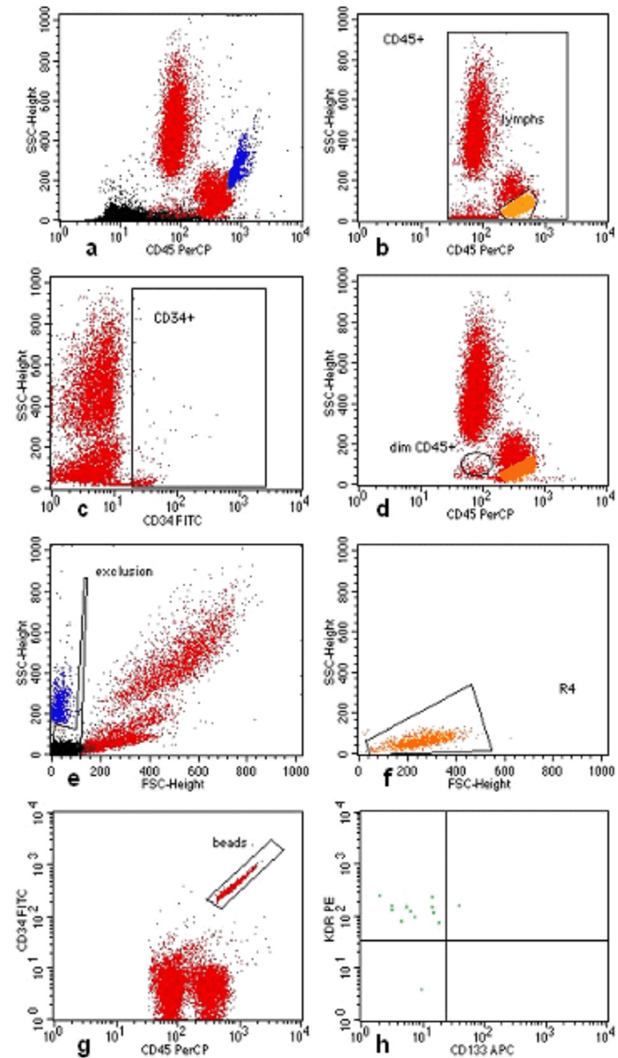

Figure 1 – ***Gating strategy for cPCs enumeration.***

Statistical analysis

Data are depicted as means ± SD. Results from flow cytometry are expressed as cells per 100 µL. Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test with Lilliefors' correlation. Differences between adjacent time points were analyzed with Wilcoxon matched-pairs signed-rank test. Spearman's correlation analysis was utilized for determining relationships between circulating level of progenitor cells and independent variables. To identify independent determinants of cPCs numbers, a multivariate linear regression analysis for various cardiovascular risk factors or other patients' characteristics (*e.g.*, infarction size) was performed. All tests were two-sided. Statistical analysis was completed using SPSS software version 15.0 for Windows 7.0. A *p*-value <0.05 was considered statistically significant.

Results

The patients' characteristics are summarized in Table 1. In one single patient, statin therapy had been initiated 24 hours prior to admission.

Table 1 – *Characteristics of the study patients*

Characteristics		Values
No. of patients (*n*)		12
Age [years]		57.25±10.82
Male gender, *n* [%]		9 (75)
BMI [kg/m^2]		26.56±4.8
Smoking, *n* [%]	*current*	4 (33.3)
	history	5 (41.7)
Hypertension, *n* [%]		9 (75)
Diabetes mellitus, *n* [%]		1 (8.3)
Hyperlipidemia, *n* [%]		5 (41.7)
Family history of CAD, *n* [%]		3 (25)
Total cholesterol [mg/dL]		213.33±48
Triglycerides [mg/dL]		107.25±40
Systolic blood pressure [mmHg]		137.5±23
Diastolic blood pressure [mmHg]		83.33±9.5
Extent of coronary disease, [%]	*1*	5 (41.7)
	2	4 (33.3)
	3	3 (25)
Myocardial classification – Topol class, *n* [%]	*1*	4 (33.3)
	2	6 (50)
	3	0
	4	2 (16.7)
	5	0
Left ventricular ejection fraction [%]		44.75±10.47
Preprocedural TIMI flow, *n* [%]	*0*	6 (50)
	3	6 (50)
TIMI flow at the end of PCI, *n* [%]	*3*	12 (100)
Myocardial blush after angioplasty, *n* [%]	*2*	5 (41.7)
	3	7 (58.3)
Statin therapy, *n* [%]		12 (100)
Aspirin/Clopidogrel, *n* [%]		12 (100)
ACE inhibitor/AT1 blocker, *n* [%]		12 (100)
B-blocker, *n* [%]		11 (91.7)

BMI: Body Mass Index; CAD: Coronary Artery Disease; TIMI: Thrombolysis In Myocardial Infarction; PCI: Percutaneous Coronary Intervention; ACE: Angiotensin Converting Enzyme; AT1: Angiotensin1 Receptor.

With the use of the four surface markers mentioned above, we identified four progenitor cell phenotypes: CD34$^+$CD45^{dim}, CD133$^+$CD34$^+$CD45^{dim}, KDR$^+$CD34$^+$ CD45^{dim} and KDR$^+$CD133$^+$CD34$^+$CD45^{dim}. There is a statistically significant increase in cPCs counts in the next days after STEMI, with a different pattern depending on their phenotype. Mature cPCs (CD34$^+$CD45^{dim}, KDR$^+$ CD34$^+$CD45^{dim}) have an undulating behavior with two fairly similar peaks, first attained around the 3rd day of admission followed by a short decrease and a new raise to the 7th day (Figure 2, Panel A: a and b). The more immature cPCs (CD133$^+$CD34$^+$CD45^{dim}, KDR$^+$CD133$^+$ CD34$^+$CD45^{dim}) have just one spike on the 3rd day (Figure 2, Panel A: c and d), afterwards almost disappear from the peripheral circulation.

For mature cPCs counts, the significant differences are between baseline and the two spikes (day 3, day 7 respectively), but not between the two peaks (Figure 2, Panel B: a and c), whereas for immature ones, the significant differences are between highest value (day 3), and initial or final counts (day 1–day 7 respectively), but not between day 1–day 7 (Figure 2, Panel B: b and d).

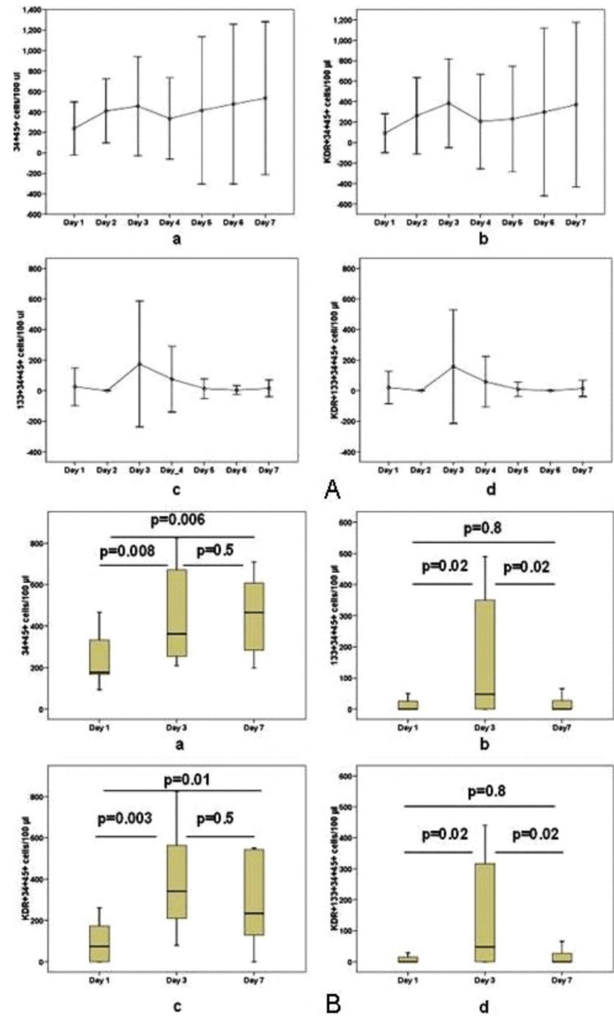

Figure 2 – ***Panel A: Mean and SD illustrating cPCs dynamics in the first seven days after STEMI. Different phenotypes are shown as follows: CD34$^+$CD45^{dim} (a), KDR$^+$CD34$^+$CD45^{dim} (b), CD133$^+$CD34$^+$CD45^{dim} (c), KDR$^+$CD133$^+$CD34$^+$CD45^{dim} (d). Panel B: cPCs counts in the key days after STEMI. Various subpopulations are presented: CD34$^+$CD45^{dim} (a), CD133$^+$CD34$^+$ CD45^{dim} (b), KDR$^+$CD34$^+$CD45^{dim} (c), KDR$^+$CD133$^+$ CD34$^+$CD45^{dim} (d). Horizontal bars and p-values spot differences between time points using Wilcoxon matched-pairs signed-rank test.***

In a bivariate correlation analysis (Table 2), we found that baseline levels of cPC (except for CD34$^+$CD45^{dim} cells) negatively correlated with preprocedural TIMI flow grade, postprocedural myocardial blush score, Topol class and Left Ventricular Ejection Fraction (LVEF) on admission. As for the 3rd day peak, the mature phenotype was also negatively correlated with myocardial blush grade, while the CD133$^+$ cells inversely associated with LVEF. None of the above-mentioned independent variables influenced the peak of cPC at the 7th day.

In a multivariate regression analysis, LVEF, TIMI flow and myocardial blush proved to be negative independent predictors for cPCs variation after STEMI, as showed in Table 3.

Table 2 – *Bivariate correlations between circulation progenitor cell number as dependent variables and myocardial characteristics*

	Preprocedural TIMI flow grade		Postprocedural myocardial blush		Topol class		LVEF	
	Rho	***P***	***Rho***	***P***	***Rho***	***P***	***Rho***	***P***
$CD34^+CD45^{dim}$ day 3			-0.86	0.01				
$CD133^+CD34^+CD45^{dim}$ day 1	-0.572	0.05	-0.75	0.03	-0.724	0.008	-0.779	0.003
$CD133^+CD34^+CD45^{dim}$ day 3							-0.617	0.032
$KDR^+CD34^+CD45^{dim}$ day 1							-0.767	0.004
$KDR^+CD34^+CD45^{dim}$ day 3			-0.86	0.01				
$KDR^+CD133^+CD34^+CD45^{dim}$ day 1	-0.570	0.05	-0.74	0.04	-0.721	0.008	-0.777	0.003
$KDR^+CD133^+CD34^+CD45^{dim}$ day 3							-0.617	0.032

TIMI: Thrombolysis In Myocardial Infarction; LVEF: Left Ventricular Ejection Fraction.

Table 3 – *Stepwise linear regression analysis of independent predictors related to cPCs kinetics*

	LVEF		Postprocedural myocardial blush		Preprocedural TIMI flow	
	β	***P***	***β***	***P***	***β***	***P***
$CD34^+CD45^{dim}$ day 3			-0.914	<0.001		
$CD133^+CD34^+CD45^{dim}$ day 1	-0.632	0.013	-0.479	0.036	-0.305	0.021
$CD133^+CD34^+CD45^{dim}$ day 3	-0.895	0.003				
$KDR^+CD34^+CD45^{dim}$ day 1	-1.371	<0.001				
$KDR^+CD34^+CD45^{dim}$ day 3			-0.876	0.004		
$KDR^+CD133^+CD34^+CD45^{dim}$ day 1	-0.632	0.013	-0.479	0.036	-0.305	0.021
$KDR^+CD133^+CD34^+CD45^{dim}$ day 3	-0.895	0.003				

cPCs: Circulating Progenitor Stem Cells; LVEF: Left Ventricular Ejection Fraction; TIMI: Thrombolysis In Myocardial Infarction.

Discussion

The end of the century revealed a new performer involved in cardiovascular repair: the endothelial progenitor cell. As a result, a series of studies has been conducted in order to explore the relationship between cardiovascular risk factors, levels of circulating EPCs and atherosclerosis progression [9, 13–15, 17, 18].

However, in the peripheral blood there is a variety of circulating progenitor cells which have been studied in relation with cardiovascular risk. $CD34^+$, KDR^+CD34^+, $CD133^+CD34^+$ or $KDR^+CD133^+CD34^+$ are among the most used surface markers combinations accompanied or not by $CD45^+$ [19] although there is a growing evidence supporting the idea of $CD45^{dim}$ fraction harboring the true EPC subpopulation [20].

On the other hand, rather few studies focused on the cPCs variation in the peripheral blood in relation with PCI [21–27], and even fewer with acute myocardial infarction [28–31], with quite mixed outcomes. The large heterogeneity concerning the cell types defined as EPCs, as well as the methods used for cell identification, led to heterogeneous results. The absence of a well-known and universally accepted marker combination and of a standardized *modus operandi* may underlie the interlaboratory variations in quantification of these cells and their correlation with different cardiovascular risk factors.

To our knowledge, this is the first study addressing the issue of all four main phenotypes of circulating progenitor stem cell profile during a seven-day hospitalization period in STEMI patients. We have already demonstrated in our prior work that the number of circulating EPCs in patients with acute coronary syndromes and positive troponin is 45% lower (p=0.012) as compared with the control group (neither cardiovascular risk factors nor ischemic heart disease); moreover, their number is further reduced as there are more cardiovascular risk factors present [14]. In our current study, we were interested in revealing the kinetic of cPCs after an important acute myocardial event (STEMI) in relation with some angiographic parameters.

First, our study showed that all four main phenotypes of circulating progenitor stem cells boosted up in the next days after STEMI, with different patterns depending on cell type (Figure 2, Panel A). Mature cPCs ($CD34^+CD45^+$, $KDR^+CD34^+CD45^+$) exhibited a wavy dynamic, with an early spike around the 3rd day after acute myocardial infarction (AMI), and a late one in the 7th day (Figure 2, Panel A: a and b).

The more immature cPCs ($CD133^+CD34^+CD45^+$, $KDR^+CD133^+CD34^+CD45^+$) have just one peak on the 3rd day (Figure 2, Panel A: c and d), afterwards almost disappearing from the peripheral circulation. Possible mechanisms of their decrease could be the loss of immature CD133 surface marker thus achieving a mature phenotype, and/or their capture in the sites of injured endothelium. Regarding the behavior of immature cPCs $CD133^+CD34^+$, $KDR^+CD133^+CD34^+$ after AMI, there is little published evidence. The only available information is provided by Massa *et al.* who demonstrated spontaneous mobilization within a few hours from the onset of AMI detectable until two months, but with no data collected between 24 hours and day 7 [29]. Two other research groups have studied the possible early mobilization of cPCs within the first 24 hours following PCI in patients with stable CVD and the counts remaining unchanged during the considered time [27], although one group has reported a transient fall in EPCs level six hours after PCI [23].

Our results provide data concordant with previous studies showing that $CD34^+$ cells increased after the onset of AMI, with the maximum value reached either on day 5 [31], or two days later, in which case they were positively correlated with the plasma levels of VEGF [28]. Ischemic tissues are known to release a wide array

of inflammatory cytokines and growth factors, many of which proved to be involved in cEPC mobilization and homing including VEGF, fibroblastic growth factor (FGF), insulin-like growth factor (IGF), angiopoietin-2 (Ang-2), stromal cell-derived factor-1 (SDF-1), stem cell factor (SCF), erythropoietin, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [6, 32–37]. Moreover, it has been proved that at least some of these angiopoietic growth factors (*e.g.*, VEGF, Ang-2) exhibit a double wave of release, with an early peak in the acute phase, and a late peak in the 7th day respectively [38]. Hence, this ample inflammatory reaction might be a fine explanation for cPCc dynamic following the onset of AMI.

In our study, beside the inflammatory response induced by AMI, the reaction to the PCI procedure itself might also be involved. There are reports about $CD34^+$ cells counts increasing in patients with stable angina undergoing angioplasty, with maximum value reached either at six hours (in the 7th day their number being significantly higher than baseline level) [21], or on day 7 after bare-metal stenting [22]. Jointly, all these data make obvious that, regardless of a discrete vascular injury and a relatively short period of ischemia, coronary angioplasty is sufficient to activate mobilization of bone marrow-derived cells.

Another factor likely to be involved in cPC mobilization is the treatment, as statins or angiotensin-converting enzyme inhibitors (ACEI) are known to augment ischemia-induced endothelial progenitor cell mobilization [39–41]. Since in our study all patients have been treated with statins and ACEI, it is possible that this treatment has also contributed to cPCs mobilization.

The last potential factor involved in the variation of cPCs number is the physiological diurnal variation [42], but this could be implicated only in the baseline cell count, since afterwards the blood was collected in the morning at the same hour, in order to diminish as much as possible the circadian variation.

Secondly, our work showed that different subpopulation of circulating progenitor cells might be influenced by different factors (*e.g.*, myocardial extent of injury, local blood circulation) depending of their phenotype: mature or immature respectively.

We identified a correlation between the magnitude of cPCs mobilization and the extent of macro- and micro-vascular injury. The more impeded myocardial perfusion was, the more cPCs were attracted at the site of injury. Preprocedural TIMI flow, postprocedural myocardial blush and LVEF proved to be independent predictors for cPCs variation in the next days after STEMI (Table 3). These results come to complete previous findings according to which LVEF [30] or TIMI perfusion grade [31] were negatively correlated to the circulating number of $CD34^+$ progenitor cells in patients with AMI undergoing primary angioplasty, thus emphasizing the idea that an important ischemic stress could trigger a higher level of cPCs.

Study limitations

First of all, our study included a limited number of patients, but it is, as we already stated, only a pilot study. Future work is needed in order to be able to discriminate between the effect of PCI, AMI and pharmacological treatment on the dynamics of circulation progenitor cells.

Conclusions

All four main phenotypes of circulating progenitor stem cells boosted up in the next days after STEMI, with a different pattern depending on cell type; preprocedural TIMI flow, postprocedural myocardial blush and LVEF proved to be independent predictors for cPCs variation in the first days after STEMI. Our study provides important data for understanding the kinetics of different progenitor cells after an acute myocardial event combined with a focal vascular injury (PCI), but there is still a long way toward fully elucidating the intimate mechanism of their release and homing, as well as their relationship with the atherosclerotic disease.

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Author contribution

MMM: Study conception and design, data acquisition, data analysis and interpretation, statistical analysis and drafting of manuscript. NO: Subject recruitment, data acquisition, drafting of manuscript. LC: Data acquisition, data analysis, critical revision of manuscript. ASU: Subject recruitment, data acquisition, statistical analysis. MD: Study conception and design, data interpretation, study supervision.

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