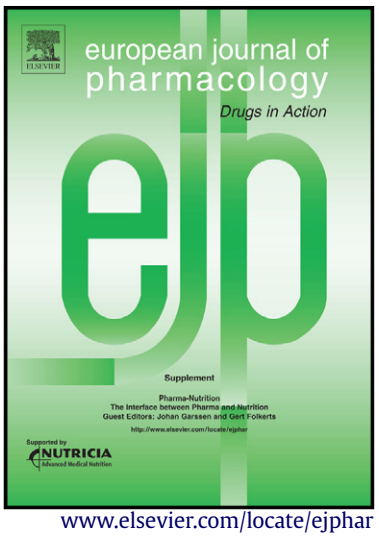


www.elsevier.com/locate/ejphar

Endothelial progenitor cells in diabetic patients with myocardial infarction - can statins improve their function?

Natália António, Ana Soares, Rosa Fernandes, Francisco Soares, Ana Lopes, Tiago Carvalheiro, Artur Paiva, Luís A Providência, Lino Gonçalves, Carlos Fontes Ribeiro

PII: S0014-2999(14)00547-0
DOI: http://dx.doi.org/10.1016/j.ejphar.2014.07.010
Reference: EJP69418

To appear in: *European Journal of Pharmacology*

Received date: 3 May 2014
Revised date: 7 July 2014
Accepted date: 9 July 2014

Cite this article as: Natália António, Ana Soares, Rosa Fernandes, Francisco Soares, Ana Lopes, Tiago Carvalheiro, Artur Paiva, Luís A Providência, Lino Gonçalves, Carlos Fontes Ribeiro, Endothelial progenitor cells in diabetic patients with myocardial infarction - can statins improve their function?, *European Journal of Pharmacology,* http://dx.doi.org/10.1016/j.ejphar.2014.07.010

TITLE PAGE

Endothelial progenitor cells in diabetic patients with myocardial infarction - can statins improve their function?

Natália António[1,2], Ana Soares[1,3], Rosa Fernandes[1,3], Francisco Soares[2], Ana Lopes[4], Tiago Carvalheiro[4], Artur Paiva[4], Luís A Providência[2], Lino Gonçalves[2], Carlos Fontes Ribeiro[1]

[1] Laboratory of Pharmacology and Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences, Faculty of Medicine, University of Coimbra, Portugal

[2] Cardiology Department, University Hospital Center of Coimbra, Portugal

[3] Center of Ophthalmology and Vision Sciences; Institute for Biomedical Imaging and Life Science, Faculty of Medicine, University of Coimbra, Portugal

[4] Blood and Transplantation Center of Coimbra | Portuguese Institute of Blood and Transplantation, Coimbra, Portugal

Corresponding author:

Natália António

Serviço de Cardiologia

Hospitais da Universidade de Coimbra/ Centro Hospitalar e Universitário de Coimbra

Praceta Mota Pinto

3000-075 Coimbra

Portugal

Telephone number: +351 239 400414, Fax number: +351 239 780552

natalia.antonio@gmail.com

ABSTRACT

The effect of statins on endothelial progenitor cells (EPCs) function derived from diabetic patients (DMpts) with acute myocardial infarction (AMI) is unknown. In this study we assess the response of early and late EPCs from diabetic versus non-diabetic patients (NDMpts) with AMI to statins.

EPCs were obtained from 10 diabetic and 10 age-matched non-diabetic male patients with AMI. For each patient, cultures of early and late EPCs were performed under 4 different conditions: normal glucose concentration (control); high glucose concentration; normal glucose concentration with atorvastatin supplementation and normal glucose concentration with pravastatin supplementation. To compare the effect of these treatments on EPC function in DMpts versus NDMpts, we performed *in vitro*: EPC colony-forming units (CFU) assay; cell cycle analysis; viability assessment and expression of the surface markers CXCR4, CD133, CD34 and KDR.

Under control conditions, CFU numbers were reduced in DMpts-derived EPCs when compared to those of NDMpts (1.4±0.8 vs 2.6±1.2 CFU/well, P=0.021). When early EPCs from DMpts were cultured in the presence of statins, CFU capacity was restored, surmounting that of NDMpts under control conditions. Statins significantly improved viability of early EPCs and delayed the onset of late EPCs senescence, even in cells from DMpts. Additionally, statins induced approximately a 2-fold increase in the proportion of late EPCs in S-phase of the cell cycle (P<0.05).

Statins have a beneficial effect on both early and late EPCs from DMpts with AMI. Despite the functional impairment of EPCs from DMpts, they exhibit similar responsiveness to statins as equivalent cells from NDMpts.

Key words: endothelial progenitor cells; diabetes mellitus; myocardial infarction; statins; function

1. Introduction

Worldwide, coronary artery disease (CAD) is the single most frequent cause of death (Steg et al., 2012). In Europe, the annual incidence of hospital admissions due to ST segment elevation acute myocardial infarction (STEMI) is estimated to be approximately 66 per 100 000 inhabitants and mortality rates caused by STEMI remain substantial (approximately 13.5% in-hospital mortality rate and 12% 6-month mortality rate) (Fox et al., 2006; Mandelzweig et al., 2006; Widimsky et al., 2010). Additionally, high-risk patients, such as diabetics, present a significantly higher mortality rate than those without diabetes (Donahoe et al., 2007). Indeed, type 2 diabetes mellitus (DM) is associated with increased risk of atherosclerotic disease and poor outcome after an acute myocardial infarction (AMI) (Donahoe et al., 2007). Nearly 50% of diabetic patients die from cardiovascular disease, establishing it as the leading cause of death among this growing population and contributing to a shortening of average life span by 5-10 years in diabetic patients (Morrish et al., 2001).

Endothelial progenitor cells (EPCs) are bone-marrow derived cells that are able to proliferate and differentiate into functionally mature endothelial cells, playing therefore an important role in the regeneration of ischemic tissue and in the maintenance of endothelium integrity (Takahashi et al., 1999). The number of circulating EPCs increases after AMI, revealing the importance of EPCs-mediated tissue and vessel repair as a "physiological" response of the organism to severe ischemia (Asahara et al., 1997; Werner et al., 2005). However, several reports have shown that EPCs are impaired, both in number and function, in diabetic patients, independently of other cardiovascular risk factors. This may, at least in part, explain the poorer outcome post-AMI in patients with DM (Fadini et al., 2007).

Several experimental and clinical studies have demonstrated that statins exert favorable effects on EPCs, independently of their lipid-lowering properties, and that this may contribute to the clinical benefit of these drugs (Dimmeler et al., 2001; Landmesser et al., 2004; Walter et al., 2002). However, it has been suggested that pravastatin is not as efficient as other HMG-CoA reductase inhibitors in improving the number and function of EPCs (Minami et al., 2009; Satoh et al., 2009) suggesting therefore that statins may differ in their ability to improve EPCs parameters.

As diabetic patients have a severely impaired endogenous pool of EPCs and no studies have yet been performed examining the direct effects of statins on EPCs isolated from diabetic patients with AMI, it is not known whether EPCs from diabetic patients are still responsive to statins stimulation.

Therefore, in this study we evaluate the *in vitro* effects of atorvastatin and pravastatin on functional parameters of early and late EPCs derived from diabetic patients with STEMI and compared them to those obtained from equivalent EPCs from non-diabetic patients. Additionally, to simulate clinical hyperglycemia, we study the effects of high glucose concentration on functional parameters of diabetic and non-diabetic derived EPCs *in vitro*.

2. Materials and methods

2.1. Study population

A prospective observational study was performed on 20 consecutive patients (10 diabetic and 10 non-diabetic patients) admitted in a single Coronary Care Unit (CCU) due to STEMI, between January 2012 and January 2013. The study was approved by the local ethics committee (project identification code: HUC-23-08). All patients gave written informed consent and research was conducted according to the principles outlined in the Declaration of Helsinki.

Patient inclusion criteria included: age from 40-70 years old, presentation to the cardiac catheterization laboratory for percutaneous coronary intervention (PCI) in the setting of STEMI within 12 h of presentation and no previous treatment with a HMG-Co-A reductase inhibitor in the last 3 months. Furthermore, only male patients were included in the study, in order to exclude the possible confounding factor of estrogens in women. Patient exclusion criteria included: active infections, clinical or biochemical evidence for the presence of concomitant overt inflammatory disease (high-sensitivity C-reactive protein > 10 mg/dl), regular use of non-steroidal anti-inflammatory drugs or anticoagulants, known auto-immune or malignant diseases, severe peripheral arterial occlusive disease, deep vein thrombosis or pulmonary embolism, patients with pacemakers, implantable cardioverter defibrillators or resynchronization devices, atrial fibrillation, recent trauma or surgery (< 1 month), recent major bleeding requiring blood transfusion (< 6 months), renal insufficiency (creatinine > 2.0 mg/dl), anemia (hemoglobin < 8.5g/dl) or thrombocytopenia (< 100 000/l), previous coronary bypass surgery, myocardial infarction within the preceding 2 months, cardiogenic shock, severe valvular disease or congenital heart disease, co-morbidities associated with a life expectancy less than 2 years and excessive alcohol consumption or illicit drugs abuse that may influence EPC kinetics. Diabetic patients already treated with insulin were also excluded. For each non-insulin treated diabetic patient with STEMI we included an age-matched non-diabetic patient.

All patients received the standard therapy for the acute phase of STEMI that included aspirin, clopidogrel and low-molecular-weight heparin, according to usual hospital practice.

On the first morning after admission, blood samples were collected from each patient for chemistry (including fasting glucose and glycosylated hemoglobin (HbA1C)) and hematological parameters determination, according to standard hospital practice.

2.2. EPCs isolation and culture

Mononuclear cells (MNCs) were isolated from 20 ml of peripheral blood, by density gradient centrifugation using Ficoll (Histopaque 1077, Sigma–Aldrich), following manufacturer's instructions. Total MNCs from each patient were cultured according to 2 different protocols to obtain early and late EPCs (see below). For each EPC culture protocol, cells were cultured under four different conditions set from the time of cell plating: 1) Control: normal glucose concentration (5.5 mM); 2) High glucose concentration (25 mM); 3) Incubation in the presence of atorvastatin (0.1 μM) and normal glucose concentration and 4) Incubation in the presence of pravastatin (10 mM) and normal glucose concentration. Statins or high glucose concentration were added at the time of isolation (day 0) and then every time the medium was changed. All experiments were performed on day 7 or day 15 for early or late EPCs, respectively.

2.2.1. Early EPCs

Immediately after isolation, $5x10^6$ MNCs were resuspended in 1.5 ml of endothelial basal medium (EBM-2, Clonetics) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing hydrocortisone, human fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), human recombinant Insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human recombinant epidermal growth factor (hEGF), gentamicin/ amphotericin B, and 20% fetal bovine serum. Cells were plated on fibronectin-coated 6-well dishes (Biocoat BD) at 37°C in a 5% CO_2 incubator. After a 48 h pre-plating step to deplete the sample of adherent macrophages and mature endothelial cells, the non-adherent cells were collected, and re-plated at a density of $1x10^6$ cells per well on fibronectin-coated 24-well plates (Biocoat BD). The medium was changed after 3 days and cells were maintained in culture until day 7.

2.2.2. Late EPCs

Late EPCs were cultured according to the method described by Fadini (Fadini et al., 2006b), with minor modifications. Briefly, MNCs were plated on 6-well fibronectin-coated plates (Biocoat BD) at a density of $5x10^6$ cells per well, and grown in EBM-2, supplemented with EGM-2-MV-SingleQuots (Clonetics) (1.5 ml per well), for 15 days. Culture medium was first changed on day 4 and then every 2 days to remove non-adherent cells. The adherent cells displayed a "cobblestone" appearance typical of endothelial cells.

2.3. Phenotypical characterization of early and late EPCs

The endothelial lineage of EPCs was confirmed by dual direct fluorescent staining with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-labelled acetylated low-density lipoprotein (acLDL; Molecular Probes) (which labels endothelial cells, via receptor-mediated endocytosis) and fluorescein isothiocyanate (FITC)-labelled Ulex europaeus agglutinin (UEA-1; Sigma) (which labels human endothelial cells via cell surface binding). At day 7 (early EPCs) or day 15 (late EPCs), adherent cells were first incubated with 10 µg/mL dil-acLDL at 37º C, for 1 h, washed three times with PBS and then fixed with 2% paraformaldehyde for 10 min. Cells were then incubated with UEA-1 (40 µg/ml) at 4ºC, for 1 h. After staining, cells were visualized using a confocal fluorescent microscope (Leica Microsystems AG, Wetzlar, Germany) and only cells displaying double positive fluorescence (dil-acLDL and UEA-1) were considered to be EPCs.

EPCs were also characterized by immunofluorescence staining for the expression of the cell-surface markers CD34 and KDR (early and late EPCs) and CD133 (early EPCs). Adherent cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma) in phosphate-buffered-saline (PBS) and incubated with phycoerythrin (PE)-Cy5-conjugated monoclonal antibodies against CD34, PE-conjugated monoclonal antibodies against KDR and allophycocyanin (APC)-conjugated monoclonal antibodies against CD133. Slides were mounted in antifading mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Burlingame, CA) for nuclear staining and examined by confocal fluorescence-microscopy.

To further confirm the endothelial phenotype of early and late EPCs, the expression of endothelial marker proteins was additionally measured by flow cytometry on day 7 or 15, respectively. Cells were detached from the culture plates by trypsinization (trypsin-EDTA 0.25%, Sigma) and labeled for 30 min at 4ºC in the dark with different fluorescent anti-human antibodies at manufacturers'

recommended concentrations. The following conjugated antibodies were used: PE-KDR (Sigma) as endothelial marker, FITC-CD34 and APC-CD133 (Miltenyi Biotec), as progenitor cell markers, APC H7-CD45 (Becton Dickinson) as a panleukocyte marker and PE-Cy5-CXCR4 (BD Pharmingen) which is critical for progenitor cell homing and embedding at sites of vascular injury (Yamaguchi et al., 2003). Data were acquired on a FACSCanto II flow cytometer (BDBioscience) and analyzed using the Flow Cytometry Software Infinicyt 1.5 (Cytognos).

2.4. In vitro studies on the effects of statins and high glucose concentration on cultured early and late EPCs

The effects of high glucose concentration, atorvastatin and pravastatin on proliferation, viability, survival, and expression of typical cell surface markers were assessed in cultured EPCs derived from diabetic and non-diabetic patients with STEMI following the protocols below:

2.4.1. Analysis of early EPCs proliferation - EPC colony-forming units (CFU) assay

Early EPCs proliferation was assessed by the number of colony-forming units, after 7 days in culture as described by Hill and colleagues (Hill et al., 2003). An EPC colony was defined as a central core of at least 50 round cells with radiating elongated spindle-like cells at the periphery. A central cluster alone without associated emerging cells was not counted as a colony. Two independent investigators under blind condition counted all colonies in each well, manually using a phase-contrast microscope at ×100 magnification, to obtain the average number of colonies per well.

2.4.2. Viability and survival evaluation

Viability of early EPCs was studied using trypan blue staining after 7 days in culture. Cultured cells from each condition were trypsinized (trypsin-EDTA 0.25%, Sigma) and the number of viable cells was counted using a hemacytometer.

Late EPCs derived from $5x10^6$ MNCs per well were quantified by direct counting, every 3 days starting from day 6 (the day of the second medium change) to day 15, and calculation of the average number of cells present in 10 randomly selected high-power fields, under a phase-contrast microscope. For this quantification ImageJ 1.45 s software (National Institutes of Health, USA) was used and survival curves created. Data are expressed as the number of cells per high-power field. At day 15, late EPCs were released from the original culture plates by trypsinization, stained with trypan blue and

counted using a hemacytometer.

2.4.3. Cell cycle analysis of late EPCs by flow cytometry – DRAQ5

Cell cycle analysis was assessed by DRAQ5 (Biostatus Ltd., Leicesterchire, UK) staining and flow cytometry evaluation. DRAQ5 specifically binds to DNA by rapidly penetrating intact cell membranes. At day 15, $3x10^5$ cells in 250 µl of PBS, previously stained for surface antigens were incubated with 2 µl of DRAQ5 for 5 min at room temperature and protected from bright light. Samples were acquired on a FACSCanto II flow cytometer (BDBiosciences). The ModFit LT™ 4.0 software (Verity Software House, Topsham, ME) was used to evaluate the proportion of late EPCs in S-phase (DNA Synthesis Phase).

2.4.4. Expression of CD34, CD133, KDR and CXCR4

Early and late EPCs were trypsinized, after 7 or 15 days in culture, respectively and analysed by flow cytometry to compare the expression of different cell surface markers (CD34, CD133, KDR and CXCR4), measured as mean fluorescence intensity (MFI).

2.5. Statistical Analysis

Statistical analyses were performed using SPSS software version 20.

Continuous variables were tested for normal distribution by Kolmogorov–Smirnov test and expressed as mean ± standard deviation or median ± interquartile range for parametric and nonparametric data, respectively. Categorical data is expressed as counts and percentages.

For comparison of continuous data unpaired Student t-tests or ANOVA tests were used when variables were normally distributed and nonparametric Mann–Whitney test or Kruskal-Wallis test for variables without a normal distribution. Categorical variables were compared with the chi-square test or with Fisher exact test as appropriate. The relationship between variables was calculated using Spearman's correlation coefficient. For all analyses, a 2-sided value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Comparison of the baseline characteristics of diabetic versus non-diabetic STEMI patients is summarized in Table 1.

The two groups were relatively homogenous in terms of age, cardiovascular risk factors and previous history of coronary artery disease (CAD). However, diabetic patients tended to be more frequently treated with ACE inhibitors/AT-1 receptor blockers and presented, as expected, significantly higher admission glycemia, fasting glycemia and HbA1c levels when compared with non-diabetic patients. In the diabetic group, the duration of diabetes varied from 3-13 years (mean 6.5 ± 3.1 years).

All 20 patients included in this study underwent successful revascularization with deployment of at least one stent.

3.2. Characterization of cultured EPCs

Both early and late EPCs displayed a characteristic EPC phenotype as judged by by dil-acLDL uptake, UEA-1 binding and expression of CD34 and VEGFR2 on the plasma membrane. Early EPCs, but not late EPCs, also expressed CD133, a marker for more immature cells. Co-staining with the nuclear marker DAPI revealed that virtually all adherent cells were dil-acLDL(+) UEA-1(+) (Fig. 1 – A and B).

Late EPCs presented a different morphology from early EPCs; after 15 days in culture, late EPCs acquired a cobblestone appearance instead of the characteristic elongated and spindle shape of early EPCs.

Flow cytometry analysis demonstrated that the majority of early and late cultured EPCs exhibited light-scattering properties consistent with a relatively large cell size. Additionally, this analysis revealed that early and late EPCs were positive for KDR, CD34 and CXCR4, confirming their endothelial phenotype. As expected, only early EPCs expressed the myeloid marker CD45 and the marker for immaturity/stemness CD133.

3.3. Effect of statins and high glucose concentration on CFUs

After 5 to 7 days in culture, attached cells exhibited typical colony-forming units, with a characteristic central cluster of round cells and peripheral radiating flat cells (Fig. 1-C). These cells showed the characteristic Dil-Ac-LDL uptake and UEA-1 lectin binding.

Under control conditions, the number of cell colonies generated from $5x10^6$ MNCs was 2.1 ± 1.2/well and incubation in high-glucose (25 mmol/l) resulted in diminished CFU formation (31% inhibition, $P<0.05$); in contrast, the number of colonies resulting from incubation with atorvastatin (3.9±1.8) or pravastatin (4.2±2.9) was significantly higher when compared to untreated cells ($P<0.001$),

corresponding to a 1.9 to 2-fold increase in colony formation when compared to control conditions (Fig. 2).

Under control conditions, CFU numbers were significantly reduced in cells derived from diabetic patients when compared to equivalent cells obtained from non-diabetic patients (1.4±0.8 versus 2.6±1.2 CFU/well, P=0.021) (Fig. 3). When cells of non-diabetic patients were grown in high glucose, CFU capacity was significantly reduced reaching similar numbers to those observed for cells derived from diabetic patients under control conditions (1.6±0.7 versus 1.4±0.8 CFU/well, respectively, P=0.631) (Fig. 3). Finally, treatment of early EPCs from diabetic patients with 0.1 μM atorvastatin or 10 mM pravastatin resulted in increased CFU values, surpassing CFU capacity of early EPCs from non-diabetic patients under control conditions (Fig. 3).

3.4. Effect of statins on EPCs survival and viability

3.4.1. Early EPCs

After 7 days in culture, the number of viable cells was significantly higher when the medium was supplemented with atorvastatin ($11.7\times10^4 \pm 4.8\times10^4$ cells/ml) or with pravastatin ($12.6\times10^4 \pm 7.8\times10^4$ cells/ml) when compared to values obtained under control conditions ($7.7\times10^4 \pm 3.5\times10^4$ cells/ml) (P=0.041). This positive effect of statins on early EPCs survival was also observed in the diabetic subgroup (atorvastatin treatment: $11.4\times10^4 \pm 3.2\times10^4$ cells/ml; pravastatin treatment: $14.1\times10^4 \pm 6.8\times10^4$ cells/ml; control condition: $6.7\times10^4 \pm 2.5\times10^4$ cells/ml; P = 0.031).

Regarding the comparison between cells derived from diabetic and non-diabetic patients, no differences in the number of viable cells were observed after 7 days in culture under control conditions ($8.4\times10^4 \pm 3.9\times10^4$ cells/ml in non-diabetic patients versus $6.7\times10^4 \pm 2.5\times10^4$ cells/ml in diabetic patients, P=0.304).

3.4.2. Late EPCs

The survival curve of late EPCs showed a general downward trend, in all conditions tested. As shown in Fig. 4, statin treatment seems to delay the onset of late EPCs senescence. Moreover, this delay was verified not only in cells from non-diabetic but also in those from diabetic patients. However, survival at the later stage of culture was not affected by statin supplementation; at day 15 no significant differences were observed in the numbers of viable cells amongst the different study conditions. Additionally, no significant differences were found between the diabetic and non-diabetic subgroups

($5.2\times10^4 \pm 3.4\times10^4$ in cells from diabetics versus $5.0\times10^4 \pm 1.7\times10^4$ cells/ml in cells obtained from non-diabetics, P=0.362).

3.5. Cell cycle analysis of late EPCs by flow cytometry – DRAQ5

As atorvastatin seemed to delay senescence onset, we examined whether that was accompanied by an increase in cell proliferation by determining the proportion of late EPCs in S-phase of the cell cycle at the end of culture time.

As shown by FACS analysis of DRAQ5 labelling, statin treatment induced nearly a 2-fold increase in the proportion of cells in S-phase when compared to control conditions (atorvastatin: 13.8±4.5%; control: 7.9±3.3%, p=0.048 and pravastatin: 15.7±1.1%; control: 7.9±3.3%, P=0.025) (Fig. 5 - A). Additionally, cells from diabetic patients under control conditions showed a significantly reduced proportion of late EPCs in S-phase at day 15 (diabetics: 9.4±4.0%; non-diabetics: 21.9±6.0%, P=0.026) (Fig. 5- B).

3.6. Expression of cell surface markers assessed by FACS (mean fluorescence intensity)

Mean fluorescence intensities (MFI) for cell surface expression of the characteristic markers CD34, KDR and CXCR4 were assessed for early and late EPCs by flow cytometry. MFI for CD133 was also determined for early EPCs.

Early EPCs under control conditions expressed the following MFI values: 11249.4 ± 3576.8 for CD34, 8023.4 ± 2387.6 for CD133, 11965.8 ± 4549.4 for KDR and 48216.3 ± 17746.4 for CXCR4. Regarding the comparison between the 4 culture conditions, flow cytometric analysis revealed similar pattern of MFI values on day 7 (Fig. 6 - A). Additionally, there were no significant differences between the MFI values obtained for cells derived from diabetic and non-diabetic patients, in any of the 4 different culture conditions.

A similar analysis of MFI for marker expression in late EPCs revealed that treatment of these cells with atorvastatin resulted in an increased expression of all surface markers tested. Additionally, cells cultivated under high glucose concentration showed a tendency for lower expression levels of CD34, KDR and CXCR4 when compared to control growth conditions; this difference was statistically significant for MFI values corresponding to CXCR4 expression (control: 73590.4 ± 18061.4; high glucose: 61516.8 ± 13601.6, P=0.049). Moreover, the expression of surface markers was significantly increased in cells treated with atorvastatin when compared to glucose-stressed EPCs (22% increase for

CD34 and CXCR4, P=0.007 and P=0.008, respectively and 26% increase for KDR, P=0.024) (Fig. 6 - B).

Comparison of MFI for marker expression in late EPCs from diabetic versus non-diabetic patients revealed different patterns. Thus, KDR expression was significantly lower in cells from diabetic patients compared to those of non-diabetic patients, when cells were cultured in either control or high glucose conditions; showing the same tendency when the medium was supplemented with atorvastatin or pravastatin (Fig. 7). CD34 expression was also significantly reduced in late EPCs from diabetics when compared to those from non-diabetics, when cultured under high glucose conditions; and showed the same trend down for the other 3 culture conditions (Fig. 7). Finally, expression of the homing marker CXCR4 showed no differences between cells derived from non-diabetic or diabetic patients under any of the culture conditions tested.

4. Discussion

The major finding of this study is the demonstration that statins have a favorable effect on functional parameters of EPCs derived from diabetic patients with STEMI, with the same magnitude as what is observed in EPCs obtained from non-diabetic patients. This indicates that, despite the functional impairment of EPCs in diabetic patients, these cells are still responsive to statin stimulation.

EPCs play a key role in the repair of damaged endothelium post-myocardial infarction, through the differentiation into mature, functional endothelial cells (Takahashi et al., 1999). These endothelial precursors are very rare in peripheral circulation, but their number significantly increases after an AMI (Massa et al., 2005; Shintani et al., 2001).

It has been recognized that when EPCs are cultured in an *ex vivo* system, two different types of EPCs become apparent, differing in their time-dependent appearance - early and late EPCs. Both subsets of EPCs contribute to vascular repair, although through different mechanisms. Late EPCs have robust proliferative potential and vessel-forming ability *in vivo*, but have no significant paracrine angiogenic effects (Sieveking et al., 2008; Yoder et al., 2007). These cells appear in culture within 7-21 days and are positive for CD34 and KDR but negative for the endothelial precursor marker CD133 and the leukocyte marker CD45 (Cheng et al., 2013; Timmermans et al., 2007). In contrast, early EPCs have not yet been characterized antigenically in peripheral circulation and do not incorporate into newly forming blood vessels but instead, seem to promote angiogenesis through paracrine mechanisms (Medina et al., 2010; Sieveking et al., 2008; Yoder et al., 2007; Ziegelhoeffer et al., 2004). These cells

form colonies within 5–7 days and are positive for endothelial (KDR) and hematopoietic markers (CD45) and for the marker of immaturity/stemness CD133 (Cheng et al., 2013).

So far, only a few studies have simultaneously studied early and late EPCs from human peripheral blood (Chen et al., 2007; Deschaseaux et al., 2007; Fadini et al., 2010; Sieveking et al., 2008) but none of these have examined the function of both cells in the context of diabetes or AMI. Therefore, it remains unclear whether EPCs from diabetic patients are dysfunctional in the context of an AMI and whether their function might improve in response to cardiovascular drugs, such as statins. To address this question, the effects of atorvastatin and pravastatin on both types of EPCs obtained from STEMI patients were examined *in vitro*. Moreover, we compared the effects of these statins on several functional parameters of EPCs from diabetic versus non-diabetic patients.

Clinical outcome post-AMI is worse in patients with DM than in non-diabetic patients (Abbott et al., 1988; Miettinen et al., 1998). It has become clear that there is a deregulation in EPC response to hypoxia and an abnormal angiogenesis in diabetic patients, as a result of reduced EPC numbers in peripheral circulation and EPC dysfunction (Churdchomjan et al., 2010; Fadini et al., 2005; Tepper et al., 2002). Therefore, the value of pharmacological therapies aiming to increase numbers of endogenous EPCs, that are dysfunctional in these patients, has been questioned (Fadini et al., 2006a; Marfella et al., 2004). In other words, can EPCs of diabetic patients recover their normal function and play their expected role in vascular repair when stimulated by cardiovascular drugs, such as statins?

The present study has shown that DM has a significant negative impact on several functional parameters of both types of EPCs *in vitro*. When compared to the non-diabetic subgroup under the same conditions, cells from diabetic patients show: 1) diminished CFU capacity in early EPCs; 2) attenuated expression of the endothelial marker KDR and of the hematopoietic progenitor marker CD34 in late EPCs (however, no differences were observed in the expression of the homing marker, CXCR4); and 3) reduced proportion of late EPCs in S-phase at the end of culture time. However, no differences were observed in the survival rates of either early or late EPCs between the two patient subgroups. Taken together, these data suggest that the functional impairment of EPCs in diabetic patients might be mainly due to problems related to their proliferative capacity, as judged by a reduction in CFU capacity for early EPCs and lower proportion of late EPCs in S-phase. CXCR4, the receptor for stromal cell–derived factor-1 (SDF-1), is highly expressed on EPCs and plays a pivotal role in their homing to ischemic sites (Mohle et al., 1998). In the hypoxia microenvironment, several

chemotactic factors initiate the homing process. SDF-1, by connecting with its receptor CXCR4, represents the major chemokine for initiating EPCs migration and promoting their engraftment to the ischemic myocardium (Gallagher et al., 2007). Hence, downregulation of CXCR4 receptor expression might contribute to limited functional neovascularization capacity of EPCs (Walter et al., 2005). In the present study, we observed similar levels of CXCR4 expression in EPCs obtained from diabetic and non-diabetic patients, suggesting that homing of EPCs to sites of vascular repair and angiogenesis is not impaired in diabetes. Finally, it seems that differences in apoptosis are not a contributing factor for EPCs impairment in diabetes, as cell survival was similar in the two patient subgroups.

Several previous studies have demonstrated that, under experimental diabetic conditions, EPCs display functional impairment, such as reduced proliferation, adhesion, migration, and incorporation into tubular structures (Churdchomjan et al., 2010; Fadini et al., 2005; Krankel et al., 2005; Tepper et al., 2002). However, it should be noted that comparison of functional parameters of early and late EPCs from diabetic patients with matched non-diabetic patients in the clinical context of an AMI had never been reported before.

Importantly, in this study, we have shown for the first time that by culturing EPCs from non-diabetic AMI patients in experimental hyperglycemic-like conditions we were able to induce EPCs dysfunction, such as reduction in CFU formation, to equivalent levels of early EPCs from diabetic patients under normal glucose conditions. In addition, despite the expression levels of the homing marker CXCR4 being similar in late EPCs from diabetic and non-diabetic patients under normal glucose conditions, long-term exposure of late EPCs to high glucose resulted in a markedly reduction of its expression. These data suggest therefore, that hyperglycemia *per se*, could at least in part, be responsible for EPC dysfunction seen in diabetes.

Many drugs with beneficial cardiovascular properties, such as statins, have been shown to positively stimulate EPC function and numbers (Dimmeler et al., 2001; Landmesser et al., 2004; Walter et al., 2002).

Large clinical trials have demonstrated that statin treatment improves prognosis in patients with AMI (Murphy et al., 2009; Pedersen et al., 2010; Schwartz et al., 2001). It is well known that HMG-CoA-reductase inhibitors have additional benefits on vascular function (known as pleiotropic effects) that are independent of their cholesterol-lowering ability (LaRosa, 2001; Palinski, 2001; Takemoto and Liao, 2001). These pleiotropic effects include: antioxidant (Fuhrman et al., 2002) and anti-

inflammatory (Plenge et al., 2002) actions, atherosclerotic plaque-stabilizing properties (Crisby et al., 2001), anticoagulant activity (Undas et al., 2001), decreased platelet aggregation (Aviram et al., 1998), inhibition of cardiac hypertrophy (Lee et al., 2002; Takemoto et al., 2001), increased nitric oxide bioavailability (Martinez-Gonzalez et al., 2001) and improvement of endothelial function (Egashira et al., 1994). Indeed, these effects might extend beyond the cardiovascular system, to include potential clinical benefits over conditions such as Alzheimer's disease (Wolozin et al., 2000), multiple sclerosis (Vollmer et al., 2004), osteoporosis (Meier et al., 2000), and cancer. HMG-CoA reductase inhibitors prevent the conversion of HMG-CoA to mevalonate, and thereby reduce mevalonate levels and its downstream products, which are essential for critical cellular functions such as membrane integrity, protein synthesis, and cell cycle progression (Wong et al., 2002). Therefore, inhibition of the mevalonate pathway by statins might reduce the risk of cancer and improve the recurrence of aggressive cancers (Boudreau et al., 2010). However, the clinical relevance of this noncardiovascular pleiotropic effect remains controversial (Dale et al., 2006).

Importantly, enhancement of circulating EPCs numbers and promotion of their mobilization to ischemic areas by statins (Dimmeler et al., 2001; Llevadot et al., 2001; Vasa et al., 2001; Walter et al., 2002) might be key mechanisms by which statins improve survival in patients with AMI. The mechanisms underlying statins stimulation of EPCs have been extensively investigated in experimental studies. Dimmeler et al have demonstrated that statins can induce the differentiation of EPCs and upregulate their numbers *in vitro* and *in vivo* through the PI3K/Akt pathway, which is known to have a key role in endothelial biology and angiogenesis (Dimmeler et al., 2001). Moreover, statins increase homing capacity of EPCs to sites of vascular injury, through the upregulation of endothelial integrin subunits α5, β1, αv and β5 (Walter et al., 2002). Finally, statins have the ability to delay EPC senescence, through a mechanism dependent on the up-regulation of telomere repeat-binding factor 2 (TRF2), which prevents telomerase dysfunction (Assmus et al., 2003). Paradoxically, a study done by Hristov *et al* indicated that chronic statin therapy significantly reduces EPCs numbers and does not modify CFU capacity in patients with CAD (Hristov et al., 2007). However, this study has several limitations, including the fact that CFU could not be measured in all patients and that the statin group exhibited a more severe degree of CAD and had a higher prevalence of diabetes that might have biased the results. Furthermore, it is worth noting that in the study performed by Hristov *et al* statins were administered to patients, whereas in the present study atorvastatin and simvastatin were tested *in vitro*.

Given the ability to precisely control testing conditions and the absence of potential environment factors interfering with statins actions, the direct exposure of EPCs to statins *in vitro* had the advantage to specifically identify the effects of statins on EPCs. Importantly, with *in vitro* assays we overcome the problem of potential interference of other cardiovascular drugs commonly used in these patients. Furthermore, we were able to evaluate at the same time two different statins.

No previous published studies have analyzed the effects of statins on EPC function from diabetic patients, with an AMI. Therefore, we tested the hypothesis that, despite their profound endogenous dysfunction, early and late EPCs from diabetic patients are still responsive to statins.

In the present study, we have shown that *in vitro* exposure of early and late EPCs from STEMI patients to atorvastatin or pravastatin results in remarkably improved cellular proliferation, with the CFU capacity and the proportion of late EPCs in the proliferative stage doubling in number when compared to values obtained under control conditions. Notably, this statin-mediated effect was also verified in cells from diabetic patients, with resulting CFU capacity surpassing that observed in EPCs from non-diabetic patients grown in normal control conditions. Furthermore, statin treatment was also able to improve the viability of early EPCs and delay the onset of late EPCs senescence, in both non-diabetic and diabetic patients. Altogether, these data provide strong evidence that statins consistently improve *in vitro* function of both types of EPCs derived from STEMI males and that abnormal EPC function associated with diabetes can be reversed by statin treatment. These novel findings suggest that the well-accepted "hyperglycemic memory" phenomenon (Ceriello, 2009; El-Osta et al., 2008) over EPCs can be reverted by statin treatment.

Our data have important clinical implications: 1) treatment with statins may be beneficial for EPC-driven vascular repair after an AMI and may improve the cardiovascular outcome of diabetic patients; 2) our data also provide a rationale for the early statin administration in AMI patients, including those with diabetes; 3) enhancement of endothelial repair with cell-based therapies, during the *in vivo* response to ischemia, may become a realistic clinical goal, once EPC dysfunction associated with diabetes is pharmacologically reverted.

4.1. Study limitations

This study has some inherent limitations that should be considered. First, the study population is relatively small. These data, however, are based on a careful selection of patients, including only statin naïve men with STEMI, thus limiting the recruitment of large numbers of patients. Indeed, it should be

noted that each diabetic patient was individually age-matched with a non-diabetic patient and each patient served as his own control, as EPCs of each patient were cultured in 4 different settings, always including a control condition. Second, our study focused on the *ex vivo* characteristics of EPCs and did not address the *in vivo* fate of these putative cells. Therefore, as the complex homeostatic mechanisms and pathways found in the whole body have not being considered in these *in vitro* studies, only clinical studies in humans can provide the final proof that statins also improve EPCs function *in vivo*. It is not possible to know if the functional improvement induced by statins on EPCs *in vitro* really translates into a benefit in terms of vascular repair and clinical outcomes of STEMI patients. Third, although we demonstrated a statin-induced improvement on EPCs function in diabetic and nondiabetic patients with STEMI, the exact molecular mechanisms mediating this effect were not under the scope of this study and still need to be investigated further. Fourth, since we only included male patients to avoid previously confirmed gender influence on EPCs biology, the present results only support a beneficial role of statins on EPCs function in male patients. Finally, we cannot extrapolate these positive effects of short-term administration of statins *in vitro* to the chronic statin therapy performed in clinical settings.

5. Conclusions

In summary, this study provides the first evidence that statins can improve EPCs dysfunction of diabetic patients with STEMI comparable to what is seen in non-diabetic patients. Statin therapy may represent an important strategy to acutely improve vascular repair and, thereby, offer a therapeutic opportunity to improve prognosis of diabetic patients with AMI.

Competing Interests

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare: no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years, or no other relationships or activities that could appear to have influenced the submitted work.

This work was supported by unrestricted research grants from Pfizer, Inc; the Portuguese Society of Cardiology; Foundation for Science and Technology (PEst-C/SAU/UI3282/2011 and COMPETE) and Faculty of Medicine of the University of Coimbra. However, the funders had no role in the study

design, data collection, data analysis, manuscript preparation, and/or publication decisions of this paper.

Authors' contributions

All authors provided substantial contributions to the conception and design of the study as well as in the analysis and interpretation of data contained within this manuscript. Natália António, Ana Soares and Rosa Fernandes performed the *in vitro* assays. Natália António prepared the initial draft of the manuscript. All authors revised the manuscript critically for important intellectual content, and read and approved the final manuscript.

Acknowledgements

The authors would like to thank Margarida Ruas, PhD (Department of Pharmacology, University of Oxford) for her critical revision of this manuscript.

References

1. Abbott, R.D., Donahue, R.P., Kannel, W.B., Wilson, P.W., 1988. The impact of diabetes on survival following myocardial infarction in men vs women. The Framingham Study. JAMA. 260, 3456-3460.
2. Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., Isner, J.M., 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 275, 964-967.
3. Assmus, B., Urbich, C., Aicher, A., Hofmann, W.K., Haendeler, J., Rossig, L., Spyridopoulos, I., Zeiher, A.M., Dimmeler, S., 2003. HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. Circ Res. 92, 1049-1055.
4. Aviram, M., Hussein, O., Rosenblat, M., Schlezinger, S., Hayek, T., Keidar, S., 1998. Interactions of platelets, macrophages, and lipoproteins in hypercholesterolemia: antiatherogenic effects of HMG-CoA reductase inhibitor therapy. J Cardiovasc Pharmacol. 31, 39-45.
5. Boudreau, D.M., Yu, O., Johnson, J., 2010. Statin use and cancer risk: a comprehensive review. Expert Opin Drug Saf. 9, 603-621.

6. Ceriello, A., 2009. Hypothesis: the "metabolic memory", the new challenge of diabetes. Diabetes Res Clin Pract. 86 Suppl 1, S2-6.
7. Chen, Y.H., Lin, S.J., Lin, F.Y., Wu, T.C., Tsao, C.R., Huang, P.H., Liu, P.L., Chen, Y.L., Chen, J.W., 2007. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. Diabetes. 56, 1559-1568.
8. Cheng, C.C., Chang, S.J., Chueh, Y.N., Huang, T.S., Huang, P.H., Cheng, S.M., Tsai, T.N., Chen, J.W., Wang, H.W., 2013. Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses. BMC Genomics. 14, 182.
9. Churdchomjan, W., Kheolamai, P., Manochantr, S., Tapanadechopone, P., Tantrawatpan, C., Y, U.P., Issaragrisil, S., 2010. Comparison of endothelial progenitor cell function in type 2 diabetes with good and poor glycemic control. BMC Endocr Disord. 10, 5.
10. Crisby, M., Nordin-Fredriksson, G., Shah, P.K., Yano, J., Zhu, J., Nilsson, J., 2001. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. Circulation. 103, 926-933.
11. Dale, K.M., Coleman, C.I., Henyan, N.N., Kluger, J., White, C.M., 2006. Statins and cancer risk: a meta-analysis. JAMA. 295, 74-80.
12. Deschaseaux, F., Selmani, Z., Falcoz, P.E., Mersin, N., Meneveau, N., Penfornis, A., Kleinclauss, C., Chocron, S., Etievent, J.P., Tiberghien, P., Kantelip, J.P., Davani, S., 2007. Two types of circulating endothelial progenitor cells in patients receiving long term therapy by HMG-CoA reductase inhibitors. Eur J Pharmacol. 562, 111-118.
13. Dimmeler, S., Aicher, A., Vasa, M., Mildner-Rihm, C., Adler, K., Tiemann, M., Rutten, H., Fichtlscherer, S., Martin, H., Zeiher, A.M., 2001. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest. 108, 391-397.
14. Donahoe, S.M., Stewart, G.C., McCabe, C.H., Mohanavelu, S., Murphy, S.A., Cannon, C.P., Antman, E.M., 2007. Diabetes and mortality following acute coronary syndromes. JAMA. 298, 765-775.

15. Egashira, K., Hirooka, Y., Kai, H., Sugimachi, M., Suzuki, S., Inou, T., Takeshita, A., 1994. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. Circulation. 89, 2519-2524.

16. El-Osta, A., Brasacchio, D., Yao, D., Pocai, A., Jones, P.L., Roeder, R.G., Cooper, M.E., Brownlee, M., 2008. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. J Exp Med. 205, 2409-2417.

17. Fadini, G.P., Albiero, M., Boscaro, E., Menegazzo, L., Cabrelle, A., Piliego, T., Federici, M., Agostini, C., Avogaro, A., 2010. Rosuvastatin stimulates clonogenic potential and anti-inflammatory properties of endothelial progenitor cells. Cell Biol Int. 34, 709-715.

18. Fadini, G.P., Miorin, M., Facco, M., Bonamico, S., Baesso, I., Grego, F., Menegolo, M., de Kreutzenberg, S.V., Tiengo, A., Agostini, C., Avogaro, A., 2005. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol. 45, 1449-1457.

19. Fadini, G.P., Sartore, S., Agostini, C., Avogaro, A., 2007. Significance of endothelial progenitor cells in subjects with diabetes. Diabetes Care. 30, 1305-1313.

20. Fadini, G.P., Sartore, S., Albiero, M., Baesso, I., Murphy, E., Menegolo, M., Grego, F., Vigili de Kreutzenberg, S., Tiengo, A., Agostini, C., Avogaro, A., 2006a. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. Arterioscler Thromb Vasc Biol. 26, 2140-2146.

21. Fadini, G.P., Sartore, S., Baesso, I., Lenzi, M., Agostini, C., Tiengo, A., Avogaro, A., 2006b. Endothelial progenitor cells and the diabetic paradox. Diabetes Care. 29, 714-716.

22. Fox, K.A., Dabbous, O.H., Goldberg, R.J., Pieper, K.S., Eagle, K.A., Van de Werf, F., Avezum, A., Goodman, S.G., Flather, M.D., Anderson, F.A., Jr., Granger, C.B., 2006. Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). BMJ. 333, 1091.

23. Fuhrman, B., Koren, L., Volkova, N., Keidar, S., Hayek, T., Aviram, M., 2002. Atorvastatin therapy in hypercholesterolemic patients suppresses cellular uptake of oxidized-LDL by differentiating monocytes. Atherosclerosis. 164, 179-185.

24. Gallagher, K.A., Liu, Z.J., Xiao, M., Chen, H., Goldstein, L.J., Buerk, D.G., Nedeau, A., Thom, S.R., Velazquez, O.C., 2007. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. J Clin Invest. 117, 1249-1259.

25. Hill, J.M., Zalos, G., Halcox, J.P., Schenke, W.H., Waclawiw, M.A., Quyyumi, A.A., Finkel, T., 2003. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med. 348, 593-600.

26. Hristov, M., Fach, C., Becker, C., Heussen, N., Liehn, E.A., Blindt, R., Hanrath, P., Weber, C., 2007. Reduced numbers of circulating endothelial progenitor cells in patients with coronary artery disease associated with long-term statin treatment. Atherosclerosis. 192, 413-420.

27. Krankel, N., Adams, V., Linke, A., Gielen, S., Erbs, S., Lenk, K., Schuler, G., Hambrecht, R., 2005. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. Arterioscler Thromb Vasc Biol. 25, 698-703.

28. Landmesser, U., Engberding, N., Bahlmann, F.H., Schaefer, A., Wiencke, A., Heineke, A., Spiekermann, S., Hilfiker-Kleiner, D., Templin, C., Kotlarz, D., Mueller, M., Fuchs, M., Hornig, B., Haller, H., Drexler, H., 2004. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. Circulation. 110, 1933-1939.

29. LaRosa, J.C., 2001. Pleiotropic effects of statins and their clinical significance. Am J Cardiol. 88, 291-293.

30. Llevadot, J., Murasawa, S., Kureishi, Y., Uchida, S., Masuda, H., Kawamoto, A., Walsh, K., Isner, J.M., Asahara, T., 2001. HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells. J Clin Invest. 108, 399-405.

31. Mandelzweig, L., Battler, A., Boyko, V., Bueno, H., Danchin, N., Filippatos, G., Gitt, A., Hasdai, D., Hasin, Y., Marrugat, J., Van de Werf, F., Wallentin, L., Behar, S., 2006. The second Euro Heart Survey on acute coronary syndromes: Characteristics, treatment, and outcome of patients with ACS in Europe and the Mediterranean Basin in 2004. Eur Heart J. 27, 2285-2293.

32. Marfella, R., Esposito, K., Nappo, F., Siniscalchi, M., Sasso, F.C., Portoghese, M., Di Marino, M.P., Baldi, A., Cuzzocrea, S., Di Filippo, C., Barboso, G., Baldi, F., Rossi, F., D'Amico, M., Giugliano, D., 2004. Expression of angiogenic factors during acute coronary syndromes in human type 2 diabetes. Diabetes. 53, 2383-2391.

33. Martinez-Gonzalez, J., Raposo, B., Rodriguez, C., Badimon, L., 2001. 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition prevents endothelial NO synthase downregulation by atherogenic levels of native LDLs: balance between transcriptional and posttranscriptional regulation. Arterioscler Thromb Vasc Biol. 21, 804-809.

34. Massa, M., Rosti, V., Ferrario, M., Campanelli, R., Ramajoli, I., Rosso, R., De Ferrari, G.M., Ferlini, M., Goffredo, L., Bertoletti, A., Klersy, C., Pecci, A., Moratti, R., Tavazzi, L., 2005. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. Blood. 105, 199-206.

35. Medina, R.J., O'Neill, C.L., Sweeney, M., Guduric-Fuchs, J., Gardiner, T.A., Simpson, D.A., Stitt, A.W., 2010. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. BMC Med Genomics. 3, 18.

36. Meier, C.R., Schlienger, R.G., Kraenzlin, M.E., Schlegel, B., Jick, H., 2000. HMG-CoA reductase inhibitors and the risk of fractures. JAMA. 283, 3205-3210.

37. Miettinen, H., Lehto, S., Salomaa, V., Mahonen, M., Niemela, M., Haffner, S.M., Pyorala, K., Tuomilehto, J., 1998. Impact of diabetes on mortality after the first myocardial infarction. The FINMONICA Myocardial Infarction Register Study Group. Diabetes Care. 21, 69-75.

38. Minami, Y., Satoh, M., Maesawa, C., Takahashi, Y., Tabuchi, T., Itoh, T., Nakamura, M., 2009. Effect of atorvastatin on microRNA 221 / 222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. Eur J Clin Invest. 39, 359-367.

39. Mohle, R., Bautz, F., Rafii, S., Moore, M.A., Brugger, W., Kanz, L., 1998. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood. 91, 4523-4530.

40. Morrish, N.J., Wang, S.L., Stevens, L.K., Fuller, J.H., Keen, H., 2001. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. Diabetologia. 44 Suppl 2, S14-21.

41. Murphy, S.A., Cannon, C.P., Wiviott, S.D., McCabe, C.H., Braunwald, E., 2009. Reduction in recurrent cardiovascular events with intensive lipid-lowering statin therapy compared with moderate lipid-lowering statin therapy after acute coronary syndromes from the PROVE IT-TIMI 22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction 22) trial. J Am Coll Cardiol. 54, 2358-2362.

42. Palinski, W., 2001. New evidence for beneficial effects of statins unrelated to lipid lowering. Arterioscler Thromb Vasc Biol. 21, 3-5.

43. Pedersen, T.R., Cater, N.B., Faergeman, O., Kastelein, J.J., Olsson, A.G., Tikkanen, M.J., Holme, I., Larsen, M.L., Lindahl, C., Szarek, M., 2010. Comparison of atorvastatin 80 mg/day versus simvastatin 20 to 40 mg/day on frequency of cardiovascular events late (five years) after acute myocardial infarction (from the Incremental Decrease in End Points through Aggressive Lipid Lowering [IDEAL] trial). Am J Cardiol. 106, 354-359.

44. Plenge, J.K., Hernandez, T.L., Weil, K.M., Poirier, P., Grunwald, G.K., Marcovina, S.M., Eckel, R.H., 2002. Simvastatin lowers C-reactive protein within 14 days: an effect independent of low-density lipoprotein cholesterol reduction. Circulation. 106, 1447-1452.

45. Satoh, M., Minami, Y., Takahashi, Y., Tabuchi, T., Itoh, T., Nakamura, M., 2009. Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease. Clin Sci (Lond). 116, 827-835.

46. Schwartz, G.G., Olsson, A.G., Ezekowitz, M.D., Ganz, P., Oliver, M.F., Waters, D., Zeiher, A., Chaitman, B.R., Leslie, S., Stern, T., 2001. Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial. JAMA. 285, 1711-1718.

47. Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Honma, T., Katoh, A., Sasaki, K., Shimada, T., Oike, Y., Imaizumi, T., 2001. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation. 103, 2776-2779.

48. Sieveking, D.P., Buckle, A., Celermajer, D.S., Ng, M.K., 2008. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol. 51, 660-668.

49. Steg, P.G., James, S.K., Atar, D., Badano, L.P., Blomstrom-Lundqvist, C., Borger, M.A., Di Mario, C., Dickstein, K., Ducrocq, G., Fernandez-Aviles, F., Gershlick, A.H., Giannuzzi, P.,

Halvorsen, S., Huber, K., Juni, P., Kastrati, A., Knuuti, J., Lenzen, M.J., Mahaffey, K.W., Valgimigli, M., van 't Hof, A., Widimsky, P., Zahger, D., 2012. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J. 33, 2569-2619.

50. Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J.M., Asahara, T., 1999. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med. 5, 434-438.
51. Takemoto, M., Liao, J.K., 2001. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol. 21, 1712-1719.
52. Takemoto, M., Node, K., Nakagami, H., Liao, Y., Grimm, M., Takemoto, Y., Kitakaze, M., Liao, J.K., 2001. Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. J Clin Invest. 108, 1429-1437.
53. Tepper, O.M., Galiano, R.D., Capla, J.M., Kalka, C., Gagne, P.J., Jacobowitz, G.R., Levine, J.P., Gurtner, G.C., 2002. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation. 106, 2781-2786.
54. Timmermans, F., Van Hauwermeiren, F., De Smedt, M., Raedt, R., Plasschaert, F., De Buyzere, M.L., Gillebert, T.C., Plum, J., Vandekerckhove, B., 2007. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol. 27, 1572-1579.
55. Undas, A., Brummel, K.E., Musial, J., Mann, K.G., Szczeklik, A., 2001. Simvastatin depresses blood clotting by inhibiting activation of prothrombin, factor V, and factor XIII and by enhancing factor Va inactivation. Circulation. 103, 2248-2253.
56. Vasa, M., Fichtlscherer, S., Adler, K., Aicher, A., Martin, H., Zeiher, A.M., Dimmeler, S., 2001. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation. 103, 2885-2890.
57. Vollmer, T., Key, L., Durkalski, V., Tyor, W., Corboy, J., Markovic-Plese, S., Preiningerova, J., Rizzo, M., Singh, I., 2004. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. Lancet. 363, 1607-1608.

58. Walter, D.H., Haendeler, J., Reinhold, J., Rochwalsky, U., Seeger, F., Honold, J., Hoffmann, J., Urbich, C., Lehmann, R., Arenzana-Seisdesdos, F., Aicher, A., Heeschen, C., Fichtlscherer, S., Zeiher, A.M., Dimmeler, S., 2005. Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res. 97, 1142-1151.

59. Walter, D.H., Rittig, K., Bahlmann, F.H., Kirchmair, R., Silver, M., Murayama, T., Nishimura, H., Losordo, D.W., Asahara, T., Isner, J.M., 2002. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation. 105, 3017-3024.

60. Werner, N., Kosiol, S., Schiegl, T., Ahlers, P., Walenta, K., Link, A., Bohm, M., Nickenig, G., 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med. 353, 999-1007.

61. Widimsky, P., Wijns, W., Fajadet, J., de Belder, M., Knot, J., Aaberge, L., Andrikopoulos, G., Baz, J.A., Betriu, A., Claeys, M., Danchin, N., Djambazov, S., Erne, P., Hartikainen, J., Huber, K., Kala, P., Klinceva, M., Kristensen, S.D., Ludman, P., Ferre, J.M., Merkely, B., Milicic, D., Morais, J., Noc, M., Opolski, G., Ostojic, M., Radovanovic, D., De Servi, S., Stenestrand, U., Studencan, M., Tubaro, M., Vasiljevic, Z., Weidinger, F., Witkowski, A., Zeymer, U., 2010. Reperfusion therapy for ST elevation acute myocardial infarction in Europe: description of the current situation in 30 countries. Eur Heart J. 31, 943-957.

62. Wolozin, B., Kellman, W., Ruosseau, P., Celesia, G.G., Siegel, G., 2000. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. Arch Neurol. 57, 1439-1443.

63. Wong, W.W., Dimitroulakos, J., Minden, M.D., Penn, L.Z., 2002. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia. 16, 508-519.

64. Yamaguchi, J., Kusano, K.F., Masuo, O., Kawamoto, A., Silver, M., Murasawa, S., Bosch-Marce, M., Masuda, H., Losordo, D.W., Isner, J.M., Asahara, T., 2003. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation. 107, 1322-1328.

65. Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T., Ingram, D.A., 2007. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood. 109, 1801-1809.

66. Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., Schaper, W., 2004. Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res. 94, 230-238.

Figure Captions

Fig. 1 Characterization of early EPCs at day 7 (A) and late EPCs at day 15 (B). Representative images are shown for cells derived from a diabetic STEMI patient cultured under control conditions. Nuclear stain DAPI (blue) was used to identify cells by fluorescence microscopy. Images (400 X) illustrate that all adherent cells were positively labelled with DiI-acetylated LDL, (Dil-acLDL) and FITC–Ulex Lectin. Almost all adherent early EPCs (A) were also positive for KDR, CD34 and CD133 staining. C) Early EPC colony-forming units (CFU) assay. Phase contrast microscopy showing CFUs from cells derived from a non-diabetic patient with STEMI after 7 days in culture, under control conditions.

Fig. 2 Comparison of colony-forming units (CFU) between the 4 different culture conditions. Boxplot representation of CFU of early EPCs under: 1) normal glucose concentration (control condition); 2) exposure to atorvastatin 0.1 μM; 3) high glucose concentration (25 mM) and 4) supplementation with pravastatin 10 mM. Box plots represent the interquartile range of values, the horizontal lines show the median, and whiskers indicate the maximum and minimum range excluding outliers. Circles represent outliers; asterisks represent extreme outliers. Kruskall-Wallis test was used for the statistical comparison between the 4 culture conditions (P value in the text box, <0.001). A further Mann Whitney U test was performed to compare two to two culture conditions. The outliers were treated as valid cases and included in the statistical analyses.

Fig. 3 Comparison of colony-forming units (CFU) capacity of cells derived from diabetics (filled bars) and non-diabetics (empty bars), grown in 4 different culture conditions. From left to right we have: 1) normal glucose concentration (control condition); 2) high glucose concentration (25 mM); 3) treatment with 0.1μM atorvastatin; and 4) treatment with 10 mM pravastatin. Box plots represent the interquartile range of values, the horizontal lines show the median, whiskers indicate the maximum and minimum range. (Mann-Whitney U test was used for the statistical comparisons).

Fig. 4 Effect of statins on survival curves of late EPCs from diabetic (left chart) and non-diabetic (right chart) patients. All cultures were initially set at the same cell density ($5x10^6$ cells per well). Every 3 days, starting from day 6, cells from 10 randomly selected high-power fields of a phase-contrast microscope, were quantified using ImageJ 1.45 s software. Blue lines indicate the mean cell number per field over time for control culture conditions (normal glucose concentration). Green lines and orange lines correspond to cell survival under atorvastatin or pravastatin treatment, respectively.

Fig. 5 Cell cycle analysis of late EPCs by DRAQ5 staining at day 15. A) Comparison of the proportion of late EPCs on phase S between the diabetic and non-diabetic subgroup (Mann Whitney U test). B) Comparison of the proportion of late EPCs in S-phase amongst the different culture conditions. Kruskall-Wallis test was used to compare all 4-culture conditions (P value in the text box, 0.022). A further Mann Whitney U test was used to evaluate statistically significant differences between each culture condition. Box plots represent the interquartile range of values, the horizontal lines show the median, and whiskers represent the maximum and minimum values.

Fig. 6 Comparison of surface markers expression, assessed by mean fluorescence intensity (MIF), between EPC cultivated under 4 different culture conditions: 1) control culture condition (normal glucose concentration) - empty bars; 2) atorvastatin supplementation - light grey bars; 3) high glucose concentration exposure - red bars; 4) pravastatin supplementation - dark grey bars. A) Comparison of CD34, KDR, CXCR4, and CD133 MIF expressed by early EPCs amongst all 4-studied culture conditions. B) Comparison of CD34, KDR, and CXCR4 MIF expressed by late EPCs amongst all 4-studied culture conditions. Box plots represent the interquartile range of values, the horizontal lines show the median, and whiskers indicate the maximum and minimum values excluding outliers. P values were calculated by the Kruskal-Wallis test for comparisons between all 4-culture conditions.

Fig. 7 Comparison of CD34 and KDR expression, assessed by mean fluorescence intensity (MIF), between late EPCs from diabetic (filled bars) and non-diabetic patients (empty bars). The comparison was performed regarding 4 different culture conditions: 1) control condition (normal glucose concentration) – left superior chart; 2) atorvastatin supplementation of the culture medium – right superior chart; 3) exposure to high glucose concentration – left inferior chart; and 4) culture supplementation with 10 mM pravastatin – right inferior chart. Box plots represent the interquartile range of values, the horizontal lines show the median, whiskers indicate the maximum and minimum values excluding outliers. Circles represent outliers. Mann Whitney U test was used for the comparison between EPCs from diabetic and non-diabetic patients.

Table 1 Legend

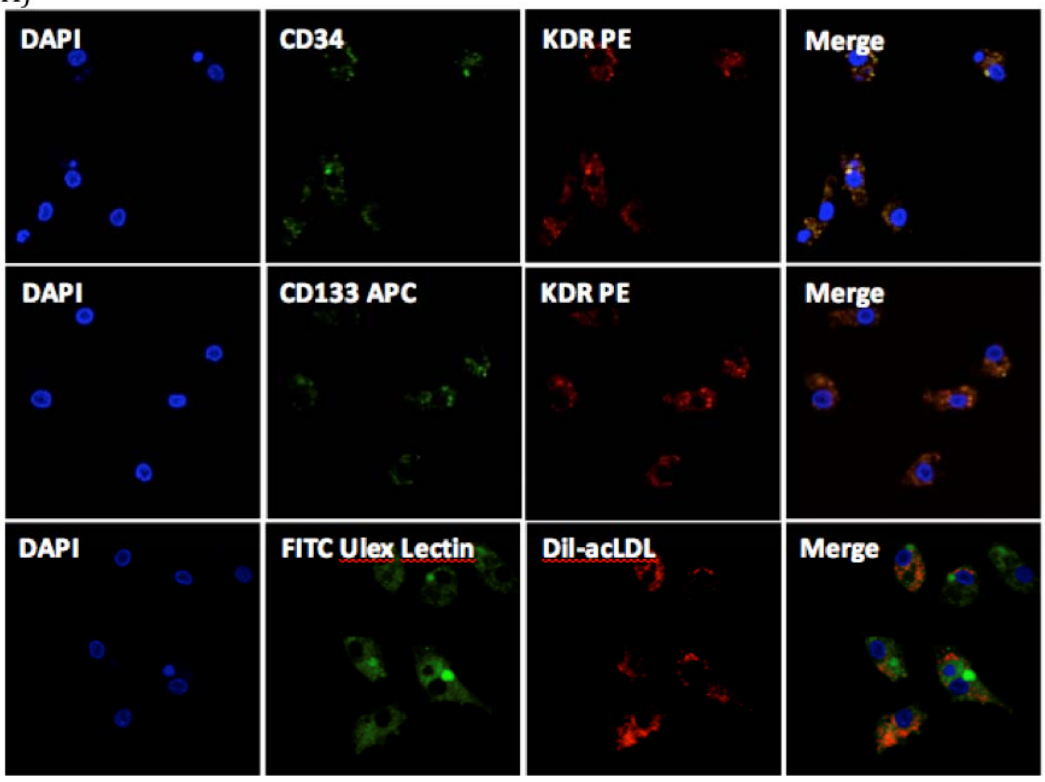
ACEI, angiotensin-converting-enzyme inhibitors; ARB, AT-1 receptor blockers; BMI, body mass index; CAD, coronary artery disease; hs-CRP, high-sensitivity C-reactive protein; PCI, percutaneous coronary intervention

Table 1 - Baseline characteristics of diabetic and non-diabetic patients with STEMI

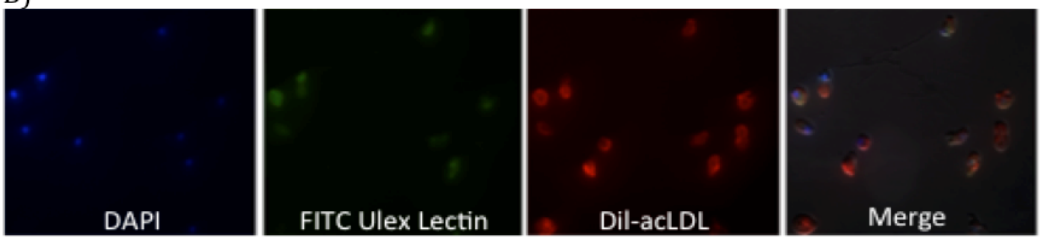
	Diabetics (N=10)	Non-diabetics (N=10)	P value
Age (years)	61.3±9.5	57.9±9.7	0.465
BMI (Kg/m^2)	27.8±2.7	27.9±4.8	0.943
Previous CAD (%)	10	10	1.000
Cardiovascular Risk Factors			
Hypertension (%)	80	60	0.628
Smoking (%)	30	50	0.653
Family history (%)	10	20	0.531
Hyperlipidemia (%)	60	60	1.000
Previous cardiovascular therapy			
Aspirin (%)	10	10	1.000
ACEI/ARB (%)	80	40	0.068
Beta-blockers (%)	10	10	1.000
Baseline laboratory			
Peak Troponin I (µg/l)	85.6±49.9	54.6±56.4	0.233
HbA1C (%)	7.4±0.9	5.5±0.5	0.001
Total cholesterol (mg/dl)	187.7±52.1	204.1±46.4	0.496
LDL cholesterol (mg/dl)	115.3±27.7	146.1±45.1	0.136
HDL cholesterol (mg/dl)	38.3±7.7	41.3±7.2	0.418
Triglycerides (mg/dl)	171.9±53.2	157.0±44.3	0.530
Admission glycemia (mg/dl)	195.1±59.5	133.6±21.8	0.006
First fasting glycemia (mg/dl)	167.8±59.5	112.5±30.0	0.016
hs-CRP at admission (mg/dl)	0.6±0.9	0.5±0.5	0.443
Peak hs-CRP (mg/dl)	4.2±1.6	3.3±3.0	0.400
Coronariography /PCI			
Number of vessels with disease	1.9±0.9	1.6±0.7	0.474
Number of stents deployed	1.6±1.1	1.5±0.8	0.755

ACEI, angiotensin-converting-enzyme inhibitors; ARB, AT-1 receptor blockers; BMI, body mass index; CAD, coronary artery disease; hs-CRP, high-sensitivity C-reactive protein; PCI, percutaneous coronary intervention

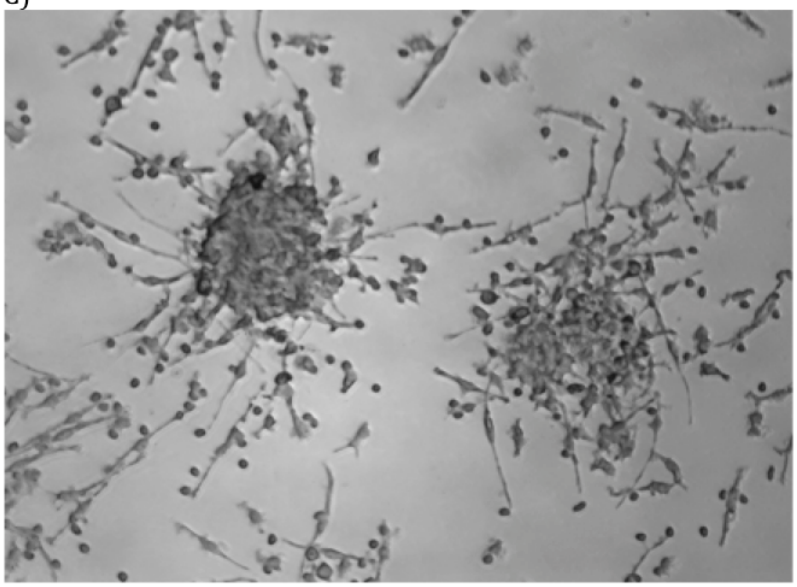
A)

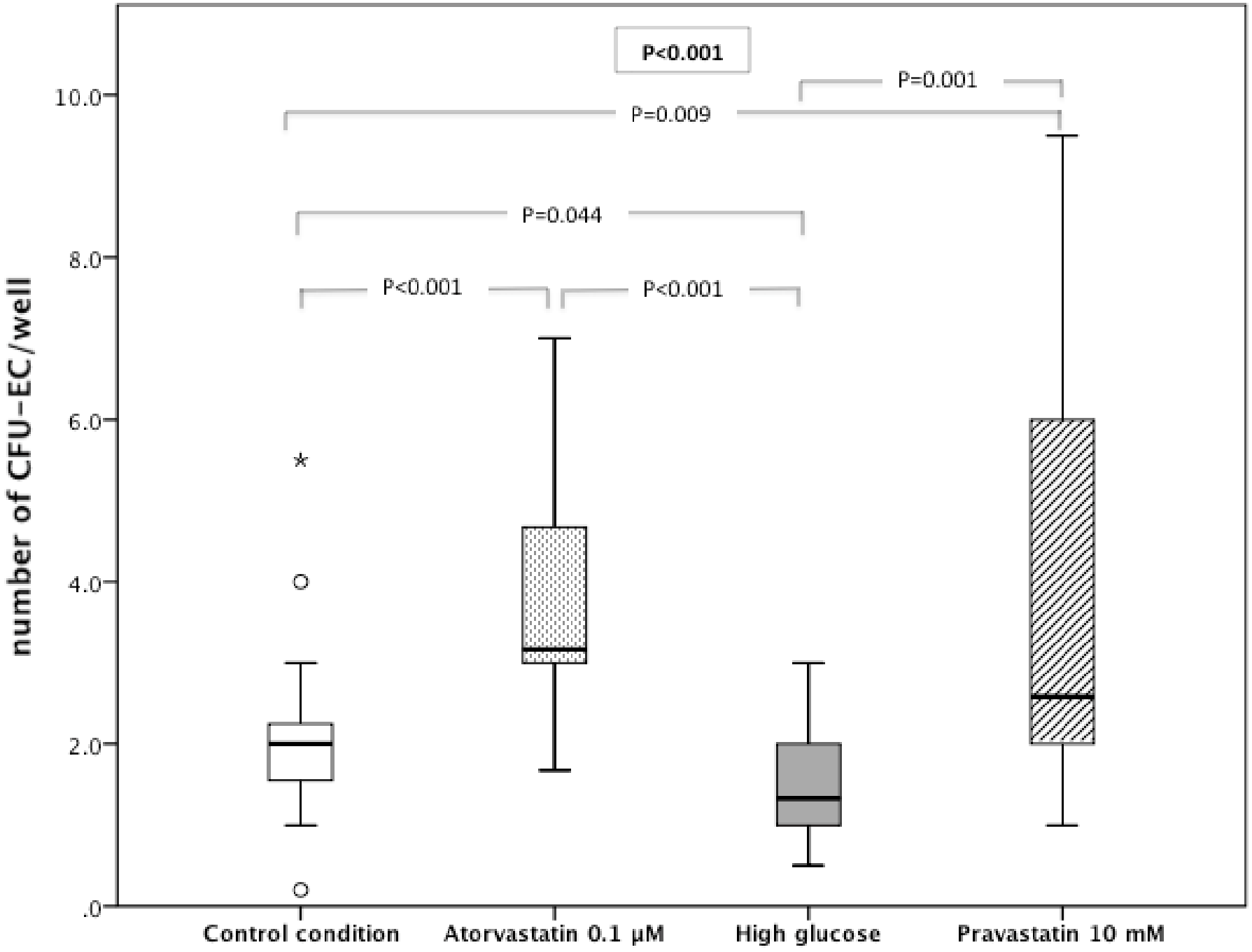

DAPI
CD34
KDR PE
Merge
DAPI
CD133 APC
KDR PE
Merge
DAPI
FITC Ulex Lectin
Dil-acLDL
Merge

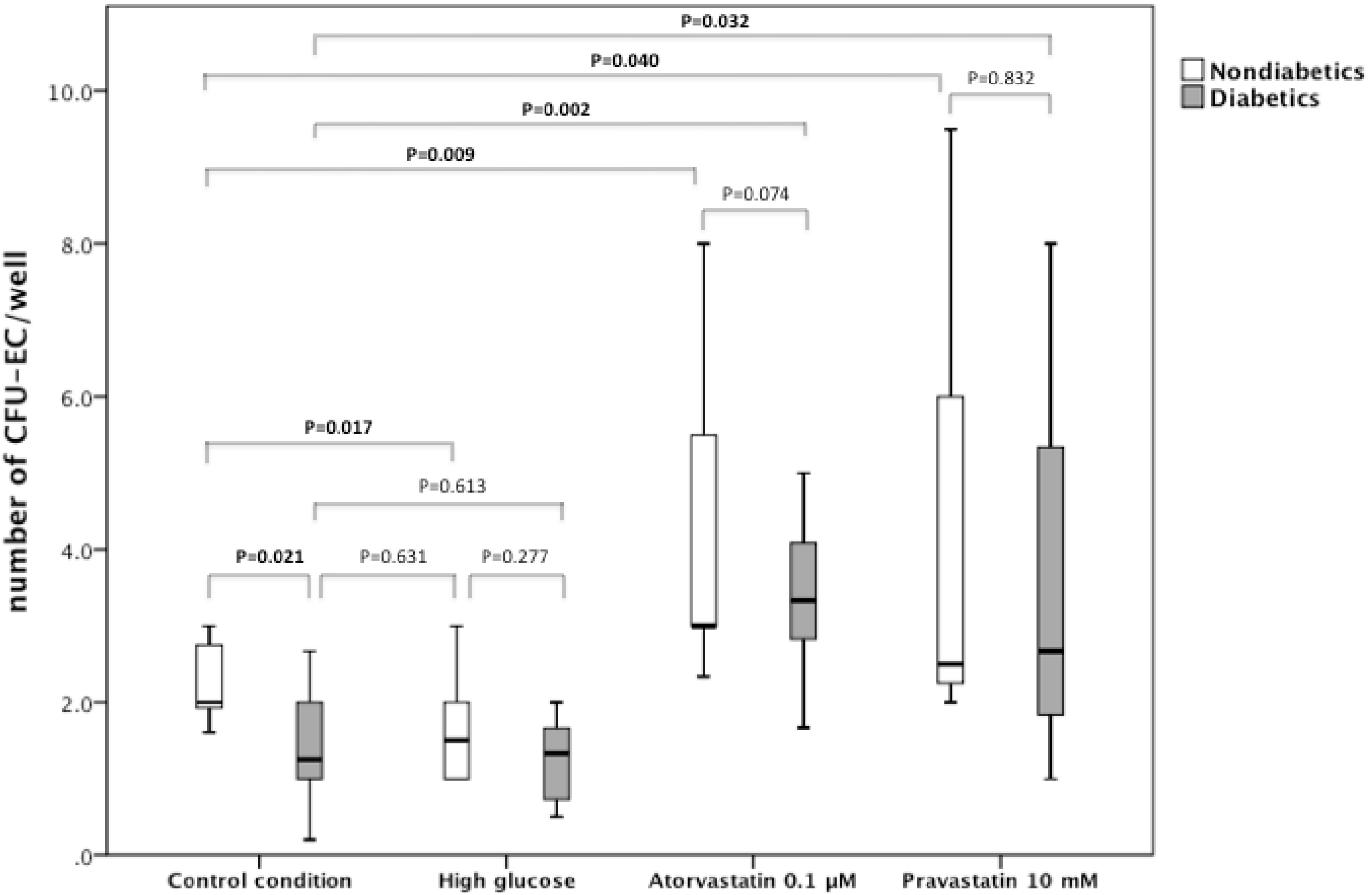
B)

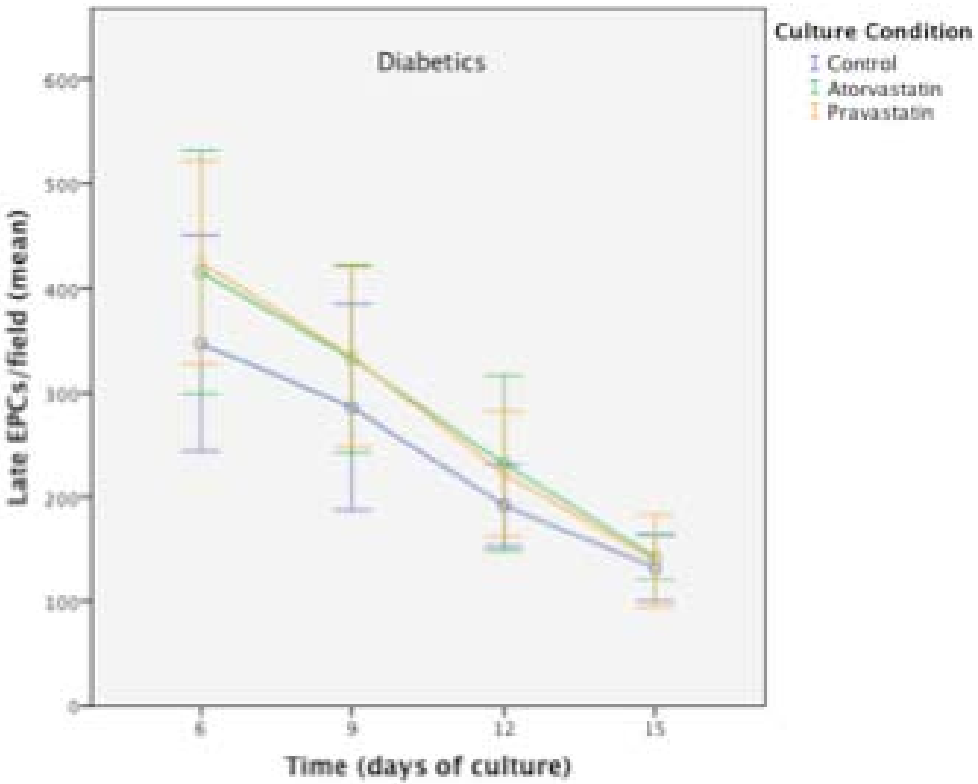

DAPI
FITC Ulex Lectin
Dil-acLDL
Merge

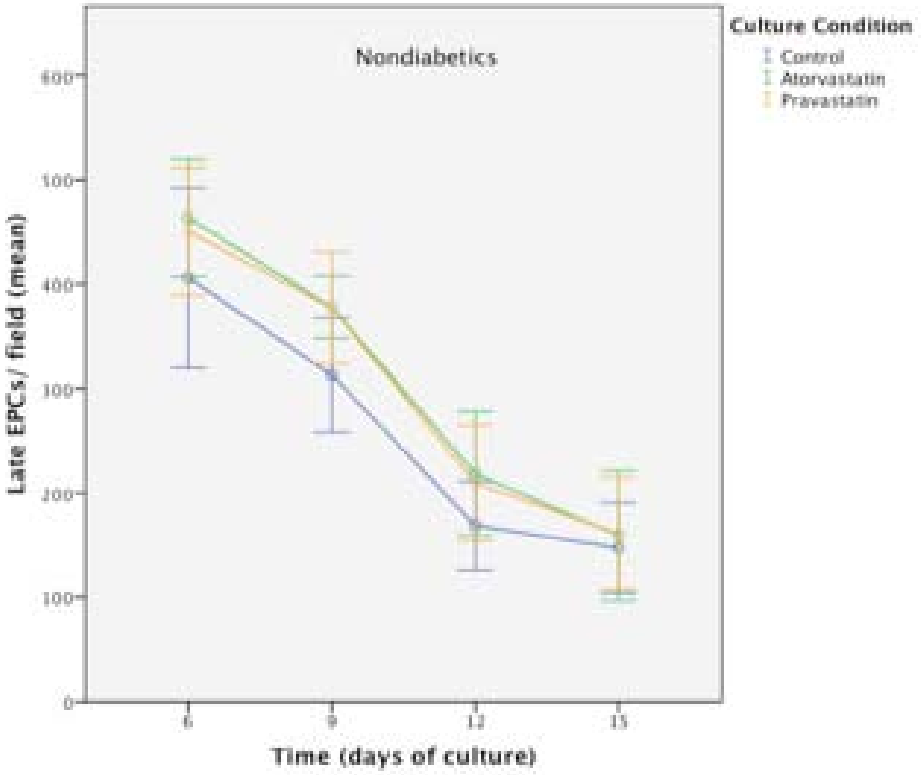
C)



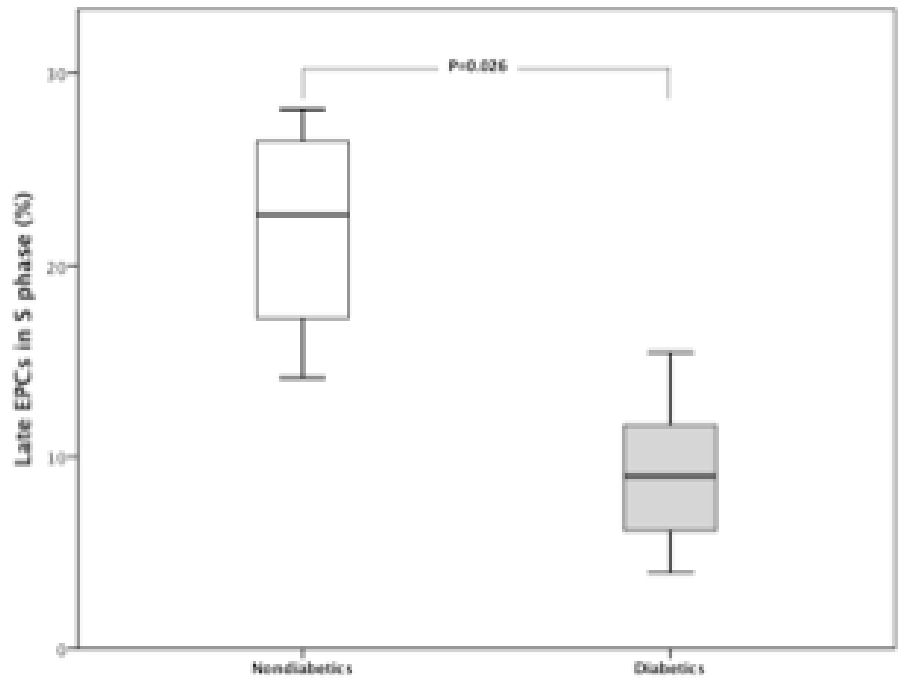

P<0.001
P=0.001
P=0.009
P=0.044
P<0.001
P<0.001
number of CFU-EC/well
10.0
8.0
6.0
4.0
2.0
.0
Control condition
Atorvastatin 0.1 µM
High glucose
Pravastatin 10 mM


P=0.032
P=0.040
P=0.832
P=0.002
P=0.009
P=0.074
P=0.017
P=0.613
P=0.021
P=0.631
P=0.277
Nondiabetics
Diabetics
number of CFU-EC/well
10.0
8.0
6.0
4.0
2.0
.0
Control condition
High glucose
Atorvastatin 0.1 µM
Pravastatin 10 mM

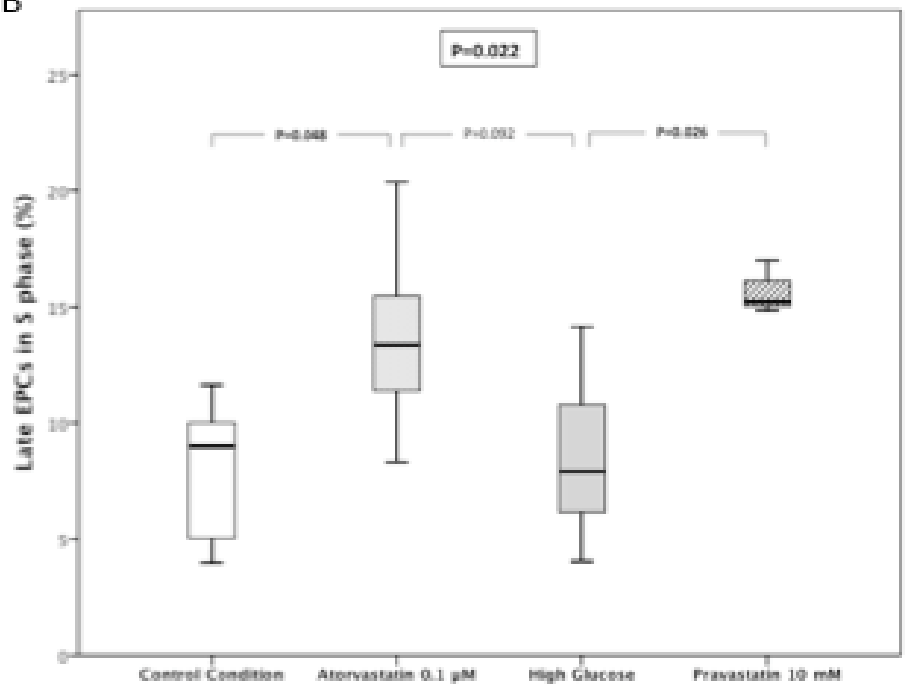

Diabetics
Culture Condition
Control
Atorvastatin
Pravastatin
Late EPCs/field (mean)
0
100
200
300
400
500
600
6
9
12
15
Time (days of culture)


Nondiabetics
Culture Condition
Control
Atorvastatin
Pravastatin
Late EPCs/ field (mean)
0
100
200
300
400
500
600
6
9
12
15
Time (days of culture)

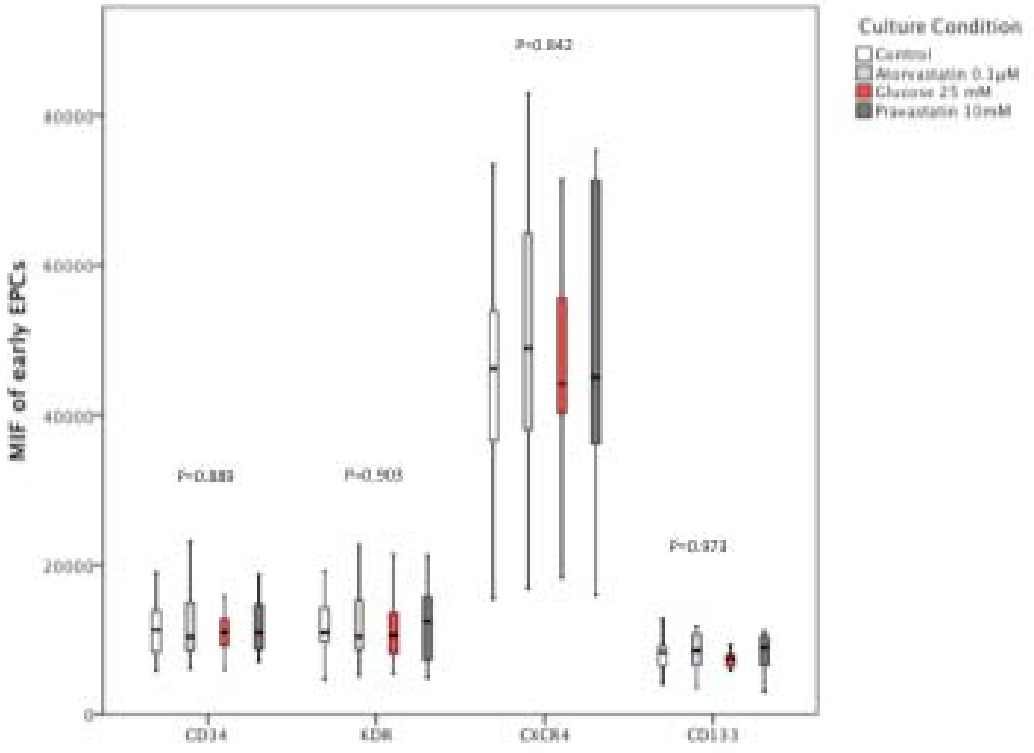
A


P=0.026
Late EPCs in S phase (%)
30
20
10
0
Nondiabetics
Diabetics

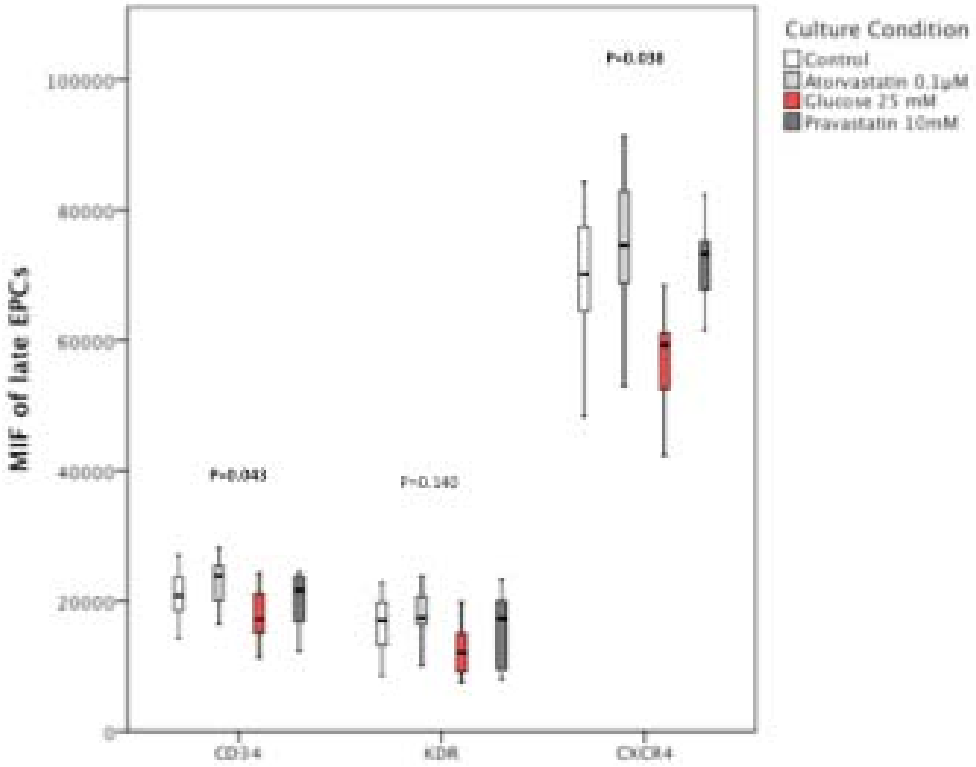
B

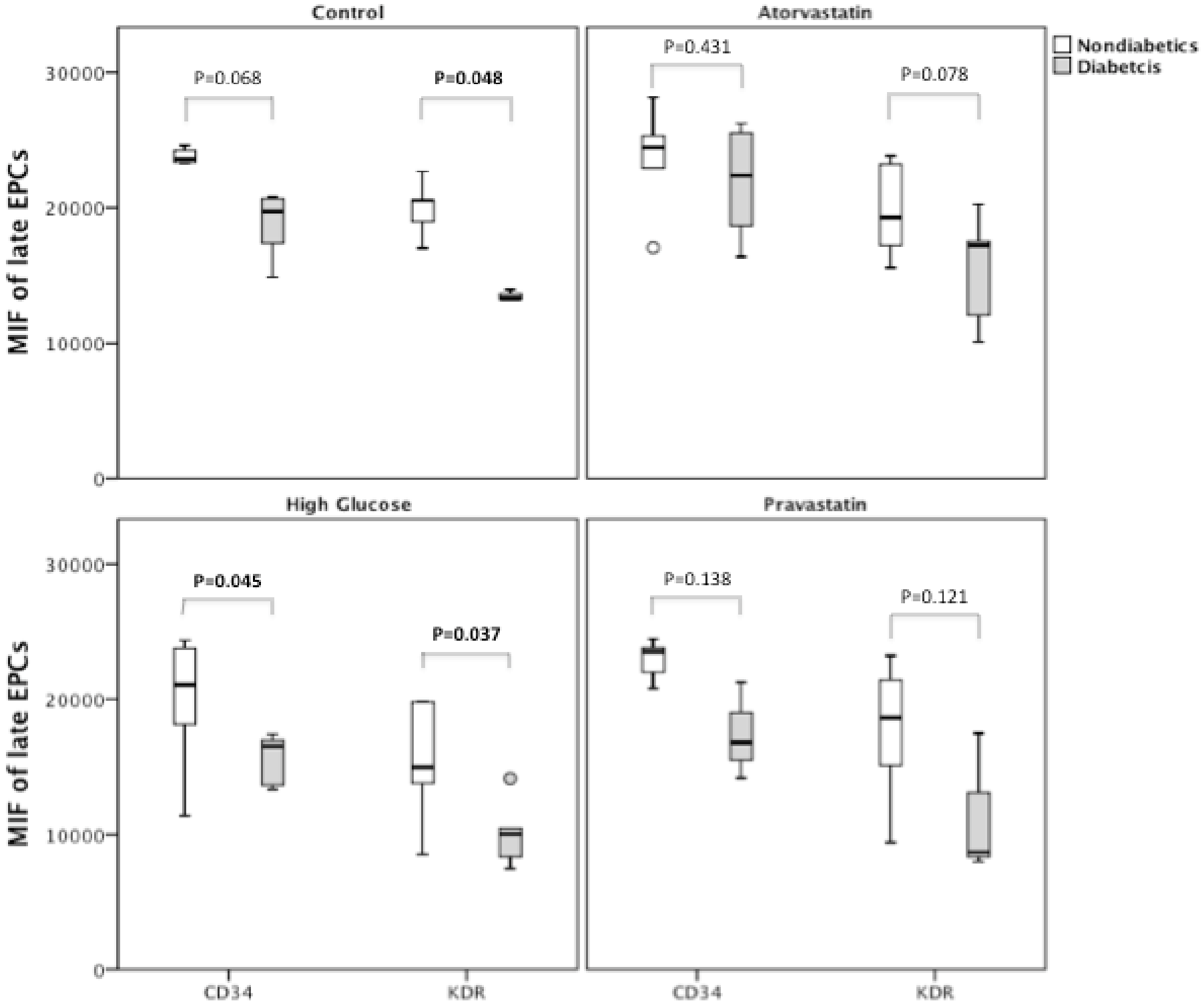

P=0.022
P=0.048
P=0.092
P=0.026
Late EPCs in S phase (%)
25
20
15
10
5
0
Control Condition
Atorvastatin 0.1 μM
High Glucose
Pravastatin 10 mM

A


MIF of early EPCs
80000
60000
40000
20000
0
P=0.889
P=0.903
P=0.042
P=0.073
CD34
KDR
CXCR4
CD133
Culture Condition
Control
Atorvastatin 0.1μM
Glucose 25 mM
Pravastatin 10mM

B


MIF of late EPCs
100000
80000
60000
40000
20000
0
P=0.043
P=0.140
P=0.038
CD34
KDR
CXCR4
Culture Condition
Control
Atorvastatin 0.1μM
Glucose 25 mM
Pravastatin 10mM


Control
Atorvastatin
High Glucose
Pravastatin
Nondiabetics
Diabetcis
MIF of late EPCs
30000
20000
10000
0
P=0.068
P=0.048
P=0.431
P=0.078
P=0.045
P=0.037
P=0.138
P=0.121
CD34
KDR
CD34
KDR