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ENDOTHELIAL PROGENITOR CELLS OF DIABETIC PATIENTS WITH ACUTE CORONARY SYNDROMES: EFFECTS OF ANTIDIABETIC AND LIPID LOWERING DRUGS

Tese de doutoramento em Ciências da Saúde, ramo de Medicina, especialidade de Ciências Fisiológicas (Farmacologia), orientada por Prof. Doutor Carlos Fontes Ribeiro, Prof. Doutor Lino Gonçalves e Doutora Rosa Fernandes e apresentada à Faculdade de Medicina da Universidade de Coimbra

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On the front cover: Representative image of phase contrast microscopy showing EPC colony-forming units from a non-diabetic patient with acute myocardial infarction, after 7 days in culture.

Tese apresentada à Universidade de Coimbra para candidatura
ao grau de Doutor em Ciências da Saúde – ramo de Medicina,
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do Prof. Doutor Lino Gonçalves e da Doutora Rosa Fernandes

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ABSTRACT

Acute coronary syndromes (ACS) represent an important public health problem. The adult human heart possesses a detectable, but limited endogenous regenerative capacity, which has attracted much interest and lead to intensive research during the past years. Endothelial progenitor stem cells (EPCs) are multipotent adult stem cells, originated from the bone marrow, that are mobilized to the peripheral circulation in response to many stimuli. EPCs play a pivotal role in postnatal neovascularization, being essential for vascular repair of the ischemic myocardium. Levels of circulating EPCs significantly increase in response to an acute myocardial infarction (AMI), highlighting the importance of EPCs-mediated repair as a "survival" response of the organism to severe ischemia. Additionally, it has been demonstrated that EPCs mobilization may also be induced by pharmacological intervention. Several drugs, including statins and many antidiabetic agents have been found to increase circulating EPCs levels and/or improve their function. Type 2 diabetes mellitus (DM) significantly worsens outcome following an ACS. Therefore, the pharmacological modulation of endogenous EPCs response to facilitate vasculogenesis in ischemic tissues is of utmost interest in this growing population. However, it is well recognized that both numbers and function of EPCs are impaired in DM, reflecting a poor endogenous regenerative capacity. This raises the question of whether pharmacological therapies based on the stimulation of endogenous EPCs are still effective in diabetic patients.

The overall goal of the present translational research was to study the potential of pharmacological stimulation of endogenous EPCs in patients with AMI, focusing on the impact of statins and insulin therapy on EPCs levels and function in diabetic patients. The specific aims were: first, to quantitatively and functionally study the EPCs response of diabetic patients to an AMI; second, to evaluate the impact of prior chronic statin therapy in circulating EPCs levels in the acute phase of the myocardial infarction (MI) and of high-intensity statin therapy at discharge on the evolution of circulating EPCs following the AMI; third, to evaluate the effects of statins on EPCs function and to assess the impact of chronic antidiabetic treatment in EPCs response to an AMI in diabetic patients.

This project was divided in two phases: 1) an *in vivo* phase, to characterize and quantify circulating EPCs in AMI patients and 2) an *in vitro* phase, to functionally evaluate the EPCs. In the first phase of this research, 100 AMI patients were prospectively included and their circulating EPCs (CD45dimCD34+KDR+, CD45dimCD133+KDR+ cells and their subpopulations co-expressing the homing marker CXCR-4) were quantified by flow cytometry (FACS), in two different moments: within the first 24 hours of admission and 3 months post-AMI. Patients were followed-up for 2 years. In the second phase of this research, circulating EPCs were obtained from 10 diabetic and 10 age-matched non-diabetic male patients with ST segment elevation AMI (STEMI). For each

patient, cultures of early and late EPCs were performed under four different conditions: 1) normal glucose concentration (control); 2) high glucose concentration; 3) normal glucose concentration with atorvastatin supplementation and 4) normal glucose concentration with pravastatin supplementation. The functional assays performed were: EPC colony-forming units (CFU); cell cycle analysis; viability assessment and expression of the surface markers CXCR4, CD133, CD34 and KDR.

The FACS analysis showed significant lower levels of circulating CD45dimCD34+KDR+ and CD45dimCD133+KDR+ EPCs in diabetic by comparison with non-diabetic AMI patients, with a parallel decrease in the subpopulations CXCR4+ ($p<0.001$). Indeed, the impaired response of EPCs to an AMI was present even in pre-diabetes and numbers of all EPCs populations were inversely correlated with glycosylated hemoglobin ($r=-0.432$, $p<0.001$ for CD45dimCD34+KDR+ EPCs). Previous chronic insulin therapy seemed to attenuate the impaired response of diabetic EPCs to the AMI. On the other hand, chronic statin pre-treatment strongly increased circulating EPCs levels in acute MI phase, even in diabetic patients. Additionally, high-intensity statin therapy at discharge prevented the expected decrease of circulating EPCs levels during follow-up. The *in vitro* study showed that despite the profound functional impairment of EPCs from diabetic AMI patients, they are still responsive to statin stimulation.

In conclusion, DM dramatically impairs the response of endogenous EPCs to an AMI, by affecting their numbers and function. This EPCs impairment is already present in pre-diabetic patients. Finally, the degree of glycemic control seems to be determinant for circulating EPCs levels and pharmacological stimulation of the endogenous EPCs seems to be a realistic goal in the treatment of AMI, even in diabetic patients.

Key words: endothelial progenitor cells, diabetes mellitus, pre-diabetes, acute myocardial infarction, statins, insulin

RESUMO

As Síndromes Coronárias Agudas (SCA) constituem um importante problema de saúde pública. O coração humano adulto possui uma capacidade regenerativa endógena detectável, mas limitada, que tem despertado muito interesse e conduzido a intensa investigação durante os últimos anos. As células progenitoras endoteliais (EPCs) são células estaminais adultas multipotentes, com origem na medula óssea, que são mobilizadas para a circulação periférica em resposta a vários estímulos. As EPCs desempenham um papel crucial na neovascularização pós-natal, sendo essenciais para a reparação vascular do miocárdio isquémico. Os níveis de EPCs circulantes aumentam significativamente em resposta a um enfarte agudo do miocárdio (EAM), salientando a importância da reparação mediada por EPCs como resposta "fisiológica" do organismo à isquemia grave. Adicionalmente, tem sido demonstrado que a mobilização das EPCs também pode ser induzida por intervenção farmacológica. Vários fármacos, incluindo estatinas e vários antidiabéticos mostraram aumentar os níveis de EPCs circulantes e/ou melhorar a sua função. A diabetes mellitus tipo 2 (DM) agrava significativamente o prognóstico após uma SCA. Portanto, a modulação farmacológica da resposta endógena das EPCs para facilitar a vasculogénese nos tecidos isquémicos é do máximo interesse nesta população crescente. No entanto, a desregulação numérica e funcional das EPCs associada à DM é bem reconhecida e traduz-se numa pobre capacidade regenerativa endógena. Isto leva-nos a questionar se o tratamento farmacológico baseado na estimulação de EPCs endógenas será eficaz mesmo em doentes diabéticos.

O objectivo global da presente investigação translacional foi estudar o potencial efeito da estimulação farmacológica das EPCs endógenas em doentes com EAM, focando no impacto das estatinas e da insulinoterapia nos níveis e função das EPCs de doentes diabéticos. Os objectivos específicos foram: em primeiro lugar, estudar quantitativa e funcionalmente a resposta das EPCs de doentes diabéticos a um EAM; em segundo lugar, avaliar o impacto do tratamento crónico prévio com estatinas nos níveis de EPCs circulantes na fase aguda do enfarte do miocárdio (EM) e do tratamento com estatina de intensidade elevada na alta hospitalar na evolução das EPCs circulantes após o EAM; em terceiro lugar, avaliar os efeitos das estatinas na função das EPCs e o impacto do tratamento antidiabético crónico na resposta das EPCs a um EAM, em doentes diabéticos.

Este projeto foi dividido em duas fases: 1) uma fase *in vivo*, para caracterização e quantificação das EPCs circulantes em doentes com EAM e 2) uma fase *in vitro* para avaliação funcional das EPCs. Na primeira fase desta investigação, foram incluídos prospectivamente 100 doentes com EAM e as suas EPCs circulantes (células CD45dimCD34+KDR+, CD45dimCD133+KDR+ e as suas subpopulações co-expressando o marcador de *homing* CXCR-4) foram quantificadas por citometria de fluxo (FACS), em dois momentos distintos: nas primeiras 24 horas da admissão e 3 meses após o EAM. Os doentes foram seguidos durante 2 anos. Na segunda fase desta investigação, obtivemos

EPCs circulantes de 10 doentes diabéticos e 10 não diabéticos, emparelhados por idade, todos do sexo masculino, com EAM com elevação do segmento ST (EAMCST). Para cada doente, realizamos culturas celulares de "early" e "late" EPCs em quatro condições distintas: 1) concentração de glicose normal (controlo); 2) elevada concentração de glicose; 3) concentração de glicose normal na presença de atorvastatina e 4) concentração de glicose normal na presença de pravastatina. Os testes funcionais realizados foram: unidades formadoras de colónias de EPCs (CFU); análise do ciclo celular; avaliação da viabilidade e expressão dos marcadores de superfície CXCR4, CD133, CD34 e KDR.

Na análise de FACS, os doentes diabéticos apresentaram níveis de EPCs CD45dimCD34+KDR+ e CD45dimCD133+KDR+ significativamente menores que os doentes não diabéticos com EAM, com redução paralela nas subpopulações CXCR4+ ($p<0,001$). Adicionalmente, observou-se desregulação da resposta das EPCs ao EAM mesmo nos doentes com pré-diabetes e os números de todas as populações de EPCs apresentaram uma relação inversamente proporcional com a hemoglobina glicosilada ($r=-0,432$, $p<0,001$ para as EPCs CD45dimCD34+KDR+). A insulinoterapia crónica prévia aparentou atenuar a desregulação da resposta das EPCs diabéticas a um EAM. Por outro lado, o pré-tratamento crónico com estatinas aumentou significativamente os níveis de EPCs circulantes na fase aguda do EM, mesmo nos doentes diabéticos. Complementarmente, o tratamento com estatina de intensidade elevada na alta hospitalar, preveniu a redução nos níveis de EPCs circulantes que seria expectável durante o período de seguimento clínico. O estudo *in vitro* mostrou que apesar do profundo distúrbio funcional das EPCs dos doentes diabéticos com EAM, estas células ainda apresentam capacidade de resposta à estimulação das estatinas.

Em conclusão, a DM prejudica drasticamente a resposta endógena das EPCs a um EAM, afectando quer os seus níveis quer a sua função. Este distúrbio das EPCs está presente mesmo nos doentes com pré-diabetes. Finalmente, o grau de controlo glicémico parece ser determinante para os níveis de EPCs circulantes e a estimulação farmacológica das EPCs endógenas parece ser um objectivo realista no tratamento do EAM, mesmo em doentes diabéticos.

Palavras-chave: células progenitoras endoteliais, diabetes mellitus, pré-diabetes, estatinas, insulina

PUBLICATIONS ARISING FROM THIS THESIS

Articles in international peer-reviewed journals:

I. **António N**, Fernandes R, Rodriguez-Losada N, Jiménez-Navarro MF, Paiva A, de Teresa Galván E, Gonçalves L, Ribeiro CF, Providência LA. Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs. Eur J Clin Pharmacol. 2010;66(3):219-30.

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III. **António N,** Fernandes R, Soares A, Soares F, Lopes A, Carvalheiro T, Paiva A, Mariano Pêgo G, Providência LA, Gonçalves L, Fontes Ribeiro C. Reduced levels of circulating endothelial progenitor cells in acute myocardial infarction patients with diabetes or pre-diabetes: accompanying the glycemic continuum. Cardiovasc Diabetol. 2014;13(1):101.

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V. **António N**, Soares A, Fernandes R, Soares F, Lopes A, Carvalheiro T, Paiva A, Mariano Pêgo G, Providência LA, Gonçalves L, Fontes Ribeiro C. Endothelial progenitor cells in diabetic patients with myocardial infarction - can statins improve their function? Eur J Pharmacol. 2014;741:25-36.

THESIS OUTLINE

This thesis is divided in four parts, whose content is summarized below.

Part I is a general introduction to the thesis, giving an overview of the state of the art in post-natal neovascularization mediated by endothelial progenitor cells, in the clinical setting of acute myocardial infarction and diabetes mellitus, with special emphasis on the potential pharmacological modulation of their endogenous response.

In **Part II**, we summarize the key research aims that will be addressed in this thesis.

Part III of this thesis contains the papers published in international peer-reviewed journals, including two systematic reviews (Chapters I and II) and three original articles (Chapters III to V).

Chapter I, *Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs*, comprises the review article that constitutes the genesis of all original research that brought light to this thesis.

In Chapter II, *Challenges in Vascular Repair by Endothelial Progenitor Cells in Diabetic Patients*, the most relevant mechanisms underlying dysfunction of endothelial progenitor cells in diabetes are reviewed.

Chapter III and IV include two original papers concerning the results of the *in vivo* study of endothelial progenitor cells in patients with acute myocardial infarction. Chapter III, comprises the manuscript *Reduced levels of circulating endothelial progenitor cells in acute myocardial infarction patients with diabetes or pre-diabetes: accompanying the glycemic continuum* and Chapter IV the manuscript *Impact of prior chronic statin therapy and high intensity statin therapy at discharge on circulating endothelial progenitor cells levels in patients with acute myocardial infarction: a prospective observational study*.

Chapter V comprises an original paper that addresses the *in vitro* part of this research, concerning the functional study of endothelial progenitor cells, *Endothelial Progenitor Cells of Diabetic Patients with Myocardial Infarction – Can Statins Improve their Function?*.

Part IV includes an integrated conclusion summarising the main results of this thesis and, since research gives answers but always raises even more questions, an outlook on potential lines of future research will be presented.

LIST OF ABBREVIATIONS

A

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitors
ACS	Acute coronary syndromes
AD	Antidiabetic
ADA	American Diabetes Association
AF	Atrial fibrillation
AGEs	Advanced glycation end products
Akt	Serine/threonine Kinase
Ang II	Angiotensin II
AMI	Acute myocardial infarction
APC	Allophycocyanin
ARBs	Angiotensin II receptor blockers
ASA	Acetylsalicylic acid
AT1	Angiotensin type 1 receptor
AT2	Angiotensin type 2 receptor
AT4	Angiotensin type 4 receptor

B

BMI	Body mass index

C

CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CCU	Coronary care unit
CD	Cluster of differentiation
CFU	Colony-forming units
CI	Confidence interval
CXCL-12	C-X-C chemokine ligand 12
CXCR-4	C-X-C chemokine receptor type 4
CV	Cardiovascular

D

DAPI	4′,6-diamidino-2-phenylindole
Dil	1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine

DM	Diabetes mellitus
DMpts	Diabetic patients
DPP-4	Dipeptidyl peptidase 4

E

EDTA	Ethylenediamine tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinases

F

FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FPG	Fasting plasma glucose
FU	Follow-up

G

G-CSF	Granulocyte-colony stimulating factor

H

HbA1c	Hemoglobin A1C / glycosylated hemoglobin
HDL	High-density lipoprotein-cholesterol
hEGF	Human recombinant epidermal growth factor
HF	Heart failure
hFGF-B	Human fibroblast growth factor-B
HIF-1	Hypoxia-inducible factor-1
HGF	Hepatocyte growth factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
hs-CRP	High sensitivity C-reactive protein

I

IFG	Impaired fasting glucose
IGF-1	Insulin-like growth factor-1
IGT	Impaired glucose tolerance
iNOS	Inducible nitric oxide synthase
ISHAGE	International Society of Hematotherapy and Graft Engineering
ITDM	Insulin-treated diabetes mellitus

L

LAD	Left anterior descending
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LVEF	Left ventricular ejection fraction

M

MACE	Major adverse cardiac events
MFI	Mean fluorescence intensity
MI	Myocardial infarction
miRNAs	microRNAs
MNCs	Mononuclear cells

N

NADPH	Nicotinamide adenine dinucleotide phosphate
NDM	Non-diabetic patients
NGM	Normal glucose metabolism
NO	Nitric oxide
NOS	Nitric oxide synthase
NSTEMI	Non-ST segment elevation myocardial infarction

O

OAD	Oral antidiabetic drugs
OGTT	Oral glucose tolerance test
oxLDL	Oxidized low-density lipoprotein

P

PBMNCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered-saline
PCI	Percutaneous coronary intervention
PE	Phycoerythrin
PI3K	Phosphatidylinositol 3-kinase
PPARγ	Peroxisome proliferator-activated receptor-γ

R

RAGE	Receptor for advanced glycation end products
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
R3-IGF-1	Human recombinant Insulin-like growth factor-1
ROS	Reactive oxygen species

S

SDF-1	Stroma-derived factor-1
STEMI	ST-segment elevation myocardial infarction

T

TIA	Transient ischemic accident
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2

U

UA	Unstable angina
UEA-1	Ulex europaeus agglutinin

V

VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2

W

WBC	White blood cells

PART I
INTRODUCTION

INTRODUCTION

Acute coronary syndromes (ACS) represent a major global public health concern. Despite the considerable progress achieved in their pharmacological and interventional treatment over the past decade, they remain a source of high morbidity and mortality worldwide[1, 2]. As a result, ACS still require continued research to improve treatment options and outcome.

The adult human heart possesses a detectable but limited endogenous regenerative capacity, which makes regenerative intervention an attractive treatment for myocardial infarction (MI)[3, 4]. Endothelial progenitor cells (EPCs) play a pivotal role in postnatal neovascularization, being essential for vascular repair of the ischemic myocardium[5-7]. Importantly, there is an intensification of EPCs mobilization from bone marrow and a markedly increase in circulating EPCs after ACS, highlighting the importance of EPCs-mediated tissue and vessel repair as a "physiological" response of the organism to severe ischemia[8, 9]. Additionally, there is a strong body of evidence showing that EPCs mobilization may be induced, not only by natural stimuli such as myocardial ischemia, but also by pharmacological agents. Several drugs, including statins[10-15], blockers of the renin-angiotensin-aldosterone system[16, 17], many antidiabetics[18-21], estrogens[22] and erythropoietin[23] have been found to increase circulating EPCs levels and/or improve their function in humans. Statins or 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have a variety of pleiotropic effects with vasculoprotective and cardioprotective activity and are among the most effective drugs to stimulate EPCs[12, 13, 15]. Hence, pharmacological stimulation of endogenous EPCs seems to be a promising strategy in the treatment of ACS patients and may bypass the need for the complexities of exogenous cell-based therapy.

Diabetes mellitus (DM) is a major independent risk factor for coronary artery disease (CAD)[24]. Furthermore, diabetic patients have a significantly worse outcome after an ACS than their non-diabetic counterparts[25-28]. Therefore, the pharmacological modulation of endogenous EPCs to facilitate vasculogenesis in ischemic tissues, in an attempt to improve outcome, is of utmost interest in this growing population. However, it is well recognized that diabetic patients present reduced numbers and dysfunctional circulating EPCs, reflecting a poor endogenous regenerative capacity. This raises important questions on the value of pharmacological therapies based on the stimulation of endogenous EPCs in diabetic patients: are EPCs from diabetic patients responsive to pharmacological modulation? Are pharmacological drugs able to completely correct their dysfunction or do they simply increase the number of dysfunctional EPCs?

1. Acute coronary syndromes and myocardial regeneration

Worldwide, CAD is the single most frequent cause of death[32]. The clinical presentations of CAD include silent ischemia, ACS, heart failure and sudden death[33]. It is well established that ACS in their different clinical presentations (ST-segment elevation myocardial infarction (STEMI), non-STEMI, and unstable angina) share a common pathophysiological substrate, that consists of rupture or erosion of a vulnerable coronary atherosclerotic plaque, with differing degrees of superimposed thrombosis and distal embolization, resulting in myocardial underperfusion[33, 34]. If the ischemic condition persists for a prolonged period of time, irreversible myocardial injury and cell death occur[35].

It was long believed that the human heart was a post-mitotic organ without any intrinsic regenerative capacity, and therefore, lost cardiomyocytes resulting from MI or normal aging process could not be replaced by newly formed cells[4, 36]. However, in the last decade robust evidence demonstrating myocardial regeneration have refuted this dogma. The study of Quaini et al., examining the chimerism of sex-mismatched transplanted heart, presented early evidence for myocardial regeneration by demonstrating active renewal of all three germ cell lineages in human hearts. This study reported that Y-chromosome-positive cardiomyocytes, vascular smooth muscle cells, and endothelial cells were identified in hearts from female donors that were transplanted into male recipients, providing direct evidence that these male primitive cells migrated from the recipient to the grafted heart[37]. Furthermore, Anversa's group has demonstrated that the human adult heart is capable of replacing its entire population of cardiomyocytes, endothelial cells and fibroblasts several times during normal life span and under physiological conditions[4, 38].

In summary, the human heart is a highly dynamic organ that retains a significant degree of intrinsic regenerative potential throughout life. However, this regenerative capacity is limited and insufficient to prevent the negative effects of myocardial infarction. Therefore, cell-based regenerative therapy and approaches for potentiating the naturally-occurring process of cardiac repair have attracted much attention during the past years, generating new hopes that cardiac regeneration might become a realistic therapeutic option for MI.

2. EPCs and postnatal neovascularization

By definition, stem cells are clonogenic cells capable of both self-renewal and differentiation into more mature cells. Classically, stem cells are divided in two broad categories: 1) pluripotent embryonic stem cells, which are derived from the inner mass of the developing embryo during the blastocyst stage, and have the potential to differentiate into any cell type of the adult body and 2) multipotent adult stem cells, which are more lineage-committed, having therefore the capacity to differentiate only into cells of a given germ layer under the appropriate stimuli[39]. EPCs are multipotent adult stem cells originated from the bone marrow that can be found circulating in the peripheral blood at very low levels under physiological conditions[40].

In a developing embryo, blood vessels are initially formed by a process known as vasculogenesis that consists in the development of new vessels from the spontaneous differentiation of bone marrow-derived mesodermal stem cells into haemangioblasts, the common precursor of haematopoietic stem cells and endothelial-lineage angioblasts[41, 42]. These immature endothelial-commited angioblasts migrate and congregate into clusters, forming the primitive vascular plexus from which a complex microcirculation arises[43]. As the embryo grows, expansion of this vascular network depends on angiogenesis, which refers to neovessel formation by *in situ* proliferation and migration of pre-existing resident mature endothelial cells[44-46].

In adults, new blood vessel formation (neovascularization) is essential for the maintenance and repair of the cardiovascular system. Historically, postnatal neovascularization was thought to occur exclusively through the mechanism of angiogenesis. However, the discovery of EPCs in 1997 by Asahara and colleagues changed this paradigm by showing that these multipotent stem cells isolated from the peripheral circulation are also capable of forming new blood vessels, even in the absence of pre-existing blood vessels (vasculogenesis)[47]. Since then, several studies have shown that these bone marrow-derived cells play a pivotal role in human vascular homeostasis and repair, by homing to sites of neovascularization and differentiating into mature endothelial cells[5-7, 48]. Circulating EPCs in the peripheral blood provide a maintenance reservoir of endothelial cells, and contribute up to 25% of endothelial cells in newly formed vessels of ischemic lesions[6, 49, 50].

The recruitment of EPCs from bone marrow to peripheral circulation and then homing to ischemic sites is a complex process regulated by many factors, including chemokines and growth factors, such as vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF) and stroma-derived factor-1 (SDF-1)[51]. Acute myocardial infarction (AMI) releases local tissue VEGF, which in turn initiates the vasculogenic cascade by activating EPCs from their quiescent state in the bone marrow, followed by their mobilization into circulation[8, 52-55]. The mobilized EPCs then travel (via a process called EPC recruitment or homing) to the sites of needed neovascularization in the ischemic tissue[6]. SDF-1α expression is also increased in heart tissue following AMI, representing the most important chemokine to initiate EPCs migration and promote their homing to the ischemic areas[55, 56]. SDF-1α mediates its effects through its specific receptor, CXCR-4 (C-X-C chemokine receptor type 4)[55, 56]. Hence, the SDF-1/CXCR-4 axis plays a key role in the response of EPCs to myocardial ischemia. There is evidence from clinical studies that circulating EPCs increase immediately after the onset of an AMI, with a subsequent peak at day 5 and a rapid decline thereafter, normalizing to baseline levels within 2 months[8, 57, 58]. This increase in circulating EPCs levels in the very early phases of an AMI confirms that myocardial ischemia is a strong stimulus for endogenous EPCs mobilization. Once at the site of tissue repair, EPCs may exert their protective effect over the ischemic myocardium via two main mechanisms: 1) by a direct action, through the differentiation in mature endothelial cells in situ and physical incorporation into new blood vessels[6, 7, 50]; or 2) indirectly through a paracrine action. There is ample evidence supporting the hypothesis that paracrine mechanisms mediated by the secretion of a broad range of cytoprotective chemokines, cytokines, and growth factors by EPCs play an essential role in vascular repair following an AMI[59-62]. A non-exhaustive list of factors known to be released by EPCs includes VEGF, hepatocyte growth factor (HGF), endothelial nitric oxide

synthase (eNOS), inducible nitric oxide synthase (iNOS), SDF-1α, and insulin-like growth factor-1 (IGF-1)[63, 64]. It is important to point out that the paracrine action might include two distinct phenomena: the first is the humoral stimulation of endogenous regeneration, and the second is the preservation of pre-existing cells. In fact, the soluble factors secreted by EPCs might activate stem cells already present in the ischemic tissue (resident stem cells), enhance EPCs proliferation, and recruit additional EPCs to the injury sites (mainly via SDF-1α, VEGF, HGF and NOS actions) and simultaneously inhibit cell death (mainly via the production of IGF-1, a potent anti-apoptotic factor)[59, 65, 66]. Of relevance, irrespective of the mechanisms involved, the final result is a protective effect on the cardiovascular system with improved blood supply to the ischemic penumbra, which in turn would augment oxygen supply, and help rescue cells from critical ischemia with a resulting decrease in the infarction area[47, 67, 68].

3. Phenotypical characterization of EPCs

The exact definition and phenotypic characterization of EPCs is still an ongoing and unresolved question. In general, two approaches have been used to isolate circulating EPCs: a) culture and colony assays and b) identification of EPCs based on surface markers by fluorescence-activated cell sorting (FACS) or flow cytometry.

Unfortunately, there is no unique or specific marker that definitely identifies EPCs. By definition, EPCs must co-express surface markers of both endothelial cells and progenitor cells, being, therefore, possible to identify and quantify circulating EPCs trough the combination of various surface markers by flow cytometry[47, 69, 70]. However, given the change in cell membrane marker profiles during the process of mobilization and maturation, definition of EPCs by surface antigens is extremely challenging[71].

When analyzing EPCs by flow cytometry, a method considered the gold standard for the quantification of these cells in peripheral blood, the minimal antigenic profile should include at least one marker for stemness/immaturity (usually CD34 and/or CD133), plus at least one marker for endothelial commitment (usually kinase insert domain receptor - KDR, also known as vascular endothelial growth factor 2 - VEGFR-2)[71-73]. It is well established that EPCs have a dynamic phenotype over time, expressing different patterns of cell surface antigens, throughout their early and late stages of maturation. CD133, a surface marker of more immature hematopoietic cells than CD34, has been used to define a very early subset of putative EPCs with recognized pro-angiogenic activity[49, 74, 75].

In order to increase the specificity for EPCs, other surface markers and combinations of several antigens have been used by several groups. The parallel analysis of CD45, which is generally considered a specific pan-leukocyte marker, seems to be mandatory to distinguish between EPCs and myeloid cells (which are CD45+ and may mimic endothelial morphology in culture) [76]. While the original putative EPCs were first described as CD45– cells, recent use of polychromatic flow

cytometers, with more channels of resolution, has revealed that the EPCs described as CD45– are in fact CD45dim[77]. Moreover, it has been demonstrated that only the fraction of CD45dim cells harbours the "true" circulating EPCs with high neovascularization capacity[71]. Additionally, the SDF-1 receptor CXCR-4, which is required for EPCs homing, has also been used by some investigators to identify EPCs with a high migration and improved neovascularization capacity[78, 79]. This superior functional activity of EPCs coexpressing CXCR-4 seems to be mainly attributed to the enhanced homing and the release of multiple pro-angiogenic cytokines[72].

The widespread interlaboratory variations in FACS methodology used to identify circulating EPCs is still a problem, making interlaboratory comparability and reproducibility a difficult task. However, to try to overcome interlaboratory discrepancies, recently, Schmidt-Lucke et al. proposed a standardized protocol for identification and quantification of circulating EPCs, adapted from the International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol. This standardized protocol seems to have a high accuracy in detecting "true" EPCs, with neovascularization potential and is now commonly used to identify circulating EPCs[71].

Regarding culture assays, a strong body of evidence convincingly demonstrates that when peripheral blood mononuclear cells are studied *in vitro*, two different types of EPCs become apparent, differing mainly in their time-dependent appearance: 1) early EPCs (also known as circulating angiogenic cells or pro-angiogenic cells) and 2) late EPCs (also referred as late outgrowth endothelial cells or endothelial colony-forming cells). Both subsets of EPCs participate in neovascularization, although through different mechanisms. Early EPCs form colonies within 5–7 days and are similar to that reported in the landmark study of Ashara et al[47]. These early EPCs have myeloid/hematopoietic characteristics and share features with immune cells, particularly monocytes/macrophages. They are positive for the endothelial (KDR), hematopoietic (CD45) and immaturity/stemness (CD133) markers and are also characterized by the ability to uptake acetylated-LDL and to bind ulex lectin[80, 81]. However, early EPCs do not incorporate into newly forming blood vessels but instead, seem to promote angiogenesis through paracrine mechanisms[75, 82-84]. In contrast, late EPCs appear in culture within 7 to 21 days, are positive for CD34 and KDR but negative for the endothelial precursor marker CD133 and the leukocyte marker CD45. These EPCs obtained from long-term cultures have robust proliferative potential and vessel-forming ability *in vivo*, but have lower cytokine release and therefore, no significant paracrine angiogenic effects[75, 82, 85, 86].

These different methods used to identify and evaluate circulating EPCs seem to have little correlation and might actually complement each other. In fact, the combination of these methods for the study of EPCs enables simultaneous quantification of circulating EPCs levels (by flow cytometry) and evaluation of the functional capacity of EPCs (trough culture assays), providing complementary information to the understanding of EPCs biology.

4. EPCs biology and type 2 diabetes mellitus

Type 2 DM has already reached epidemic proportions in developed countries, becoming one of the leading causes of death and the majority of these deaths are associated with cardiovascular diseases [87, 88]. Furthermore, following MI, diabetic patients present a significantly worse prognosis compared with non-diabetic equivalents[25, 26].

DM is a metabolic condition that strongly affects EPCs. There is solid evidence that both numbers and function of EPCs are impaired in diabetic patients[29-31, 89]. Tepper et al. have convincingly demonstrated that EPCs isolated from type 2 diabetic patients displayed reduced proliferation, adhesion and incorporation into tubular structures *in vitro*[29]. Additionally, it has been experimentally shown that high glucose hampers proliferation and survival of EPCs isolated from healthy donors[90, 91]. However, despite the evidence of dysfunctional EPCs in DM, it remains unclear whether this critical dysfunction is mediated by chronic hyperglycemia or is inherent to type 2 DM *per se*.

The mechanisms underlying circulating EPC levels reduction in diabetes are still poorly understood. As the number of circulating EPCs is closely dependent on the balance between mobilization from the bone marrow and survival in the peripheral circulation, a defective mobilization could explain the reduced circulating levels of EPCs in diabetic patients. An alternative explanation for the reduced EPCs counts could be a shortening in peripheral EPCs survival.

Fadini et al. have shown an impaired EPCs mobilization and a defective compensatory angiogenesis after ischaemia-reperfusion injury in animal models of diabetes[92]. However, there are only a few studies in the literature addressing the dynamics of circulating EPCs numbers in the clinical setting of AMI[8, 9]. Despite the logical expectation of poorer bone marrow mobilization and lower levels of circulating EPCs in diabetic patients with AMI, when compared with their non-diabetic counterparts, data regarding circulating EPCs in diabetic patients with AMI are even scarcer[57, 58].

Of relevance, reduced EPCs levels have been proposed as a surrogate marker for vascular dysfunction, which independently predicts cardiovascular events in patients with cardiovascular disease [93, 94]. In the clinical setting of AMI, reduced circulating levels of EPCs and impaired migratory activity have also been associated with poorer clinical cardiovascular outcomes[95]. Furthermore, the reduction in circulating EPCs levels has been associated with the pathogenesis of vascular complications in diabetes[30].

Both the decreased number of circulating EPCs and their impaired function are likely to have a negative impact on vascular integrity and regenerative potential and might, therefore contribute to the adverse outcomes of diabetic patients. The quantitative study of the EPCs pool and the evaluation of the relative levels of different subsets of EPCs (according to their maturation stage) in diabetic patients with AMI, is of major interest. This information could contribute to a better understanding of the response of diabetic patients to myocardial ischemia and to a clarification of the underlying mechanisms.

In the natural history of DM, pre-diabetes appears as an intermediate stage between normal glucose metabolism (NGM) and overt DM and it is also associated with increased risk of cardiovascular events[96, 97]. However, there is a lack of studies investigating circulating EPCs in patients with pre-diabetes and therefore, their response to an AMI remains unknown.

5. EPCs as a target for pharmacological stimulation

Despite some controversy regarding the definition of EPCs, the literature is remarkably consistent in attributing to EPCs a critical role in endothelial maintenance and repair[5-7, 47, 55, 98]. However, as EPCs are found in limited numbers in the peripheral circulation, the native response is often insufficient to ensure an adequate neovascularization without additional intervention. Therefore, ways to enhance the regenerative response of EPCs are intuitively appealing and have attracted great interest over the past decade. The goal of improving EPCs to facilitate neovascularization in cardiovascular disease can be reached, either by cardiac cell-based regenerative therapies or through the stimulation of the endogenous pool of circulating EPCs. Although the concept of exogenous stem cell-based therapy for myocardial ischemia is straightforward in theory, it is extraordinarily complex and has been hampered by several important practical limitations. In fact, cardiac cell-based therapy remains challenged by inconsistent and, overall, modest efficacy, disappointingly poor cell engraftment to the therapeutic target zone and long-term safety concerns[99]. Hence, further pre-clinical research is clearly warranted before clinical use of cell-based regenerative therapies can be considered.

The stimulation of the endogenous EPCs response appears, therefore as an attractive alternative therapeutic strategy for myocardial infarction that may overcome the limitations of exogenous cardiac cell-based therapies.

Over the past decade several drugs have been shown to be effective in either enhancing peripheral EPCs levels or improving EPCs function[10-13, 15-21, 23]. Particularly, statins exhibit convincing beneficial effects on EPCs through multiple mechanisms, including enhancement of proliferation and differentiation[10-12], stimulation of EPCs mobilization from the bone-marrow[10, 13], improvement of migratory capacity[10, 13, 15], anti-apoptotic effects[12], and increased of EPCs homing[14].

Statins are potent inhibitors of cholesterol biosynthesis with unequivocal benefits in secondary prevention after AMI[100]. Therefore, current guidelines recommend intensive statin therapy early after admission in all AMI patients, without contraindication or history of intolerance, regardless of initial cholesterol levels[32]. The favorable effects of statins extend beyond their cholesterol lowering effects, to include so-called pleiotropic effects. These cholesterol-independent effects include, among others, antioxidant and anti-inflammatory actions, atherosclerotic plaque-stabilizing properties, anticoagulant activity, decreased platelet aggregation, inhibition of cardiac hypertrophy, increased nitric oxide bioavailability, improvement of endothelial function

and stimulation of EPCs[10-13, 101]. Since mevalonic acid, the product of HMG-CoA reductase reaction, is the precursor not only of cholesterol but also of nonsteroidal isoprenoid compounds, a suggested mechanism for these pleiotropic effects is the inhibition of isoprenoid synthesis by HMG-CoA reductase inhibitors, which leads to the inhibition of important intracellular signaling molecules such as Rho, Ras and Rab[101].

The beneficial effects of short-term statin therapy on EPCs biology have been demonstrated in patients with stable coronary artery disease[10, 71]. However, there are few and controversial clinical data concerning the long-term effects of treatment with statins on these EPCs. In fact, paradoxically, Hristov et al have verified that the administration of statins for more then 4 weeks significantly reduced circulating EPC levels in CAD patients[102]. One the other hand, despite the fact that it is expectable that statins also improve the EPCs response to an AMI, unfortunately, to date, no studies have evaluated the impact of previous long-term statin therapy on EPCs in AMI patients. Likewise, dose-dependent effects of a continuous statin therapy on EPCs in AMI patients have not yet been analyzed.

It is tempting to speculate that the mechanisms underlying the positive effects of statins on EPCs are also operative in patients with DM. However, due to the profound impairment of the endogenous EPCs pool in diabetic patients, and since no studies examining the direct effects of statins on human EPCs have yet been performed, it is important to consider the possibility that diabetic EPCs might be refractory to pharmacological stimulation.

Cumulative evidence indicates that levels of circulating EPCs are closely related to the glycemic control of diabetic patients, represented by hemoglobin A1C (HbA1c) levels, thus suggesting that hyperglycemia is a key factor in the development of EPCs impairment[103-105]. Therefore, it can be hypothesized that tight glycemic control, in general and specifically during AMI, might lead to a better response of EPCs to myocardial ischemia. Nevertheless, it has been suggested that hyperglycemia induces phenotypic changes in cells and vascular dysfunction that persist after normalization of glucose levels[106]. This phenomenon has been called “hyperglycemic memory” or “metabolic memory” and the hypothesis that it might also produce persistent EPCs dysfunction despite an intensive glycemic control has not been ruled out. In this regard, Loomans et al. reported that dysfunction of EPCs from type 1 diabetic patients were maintained, despite culturing these EPCs under normoglycemic conditions[107]. The concept of hyperglycemic memory (and the ability to reverse it) may, therefore, be an important factor in determining the success of pharmacological interventions directed towards improving EPCs in diabetic patients.

Many oral antidiabetic drugs (OAD) and insulin therapy have demonstrated significant EPC-stimulating effects. Previous clinical studies have shown that metformin (alone or in combination with glicazide)[108], peroxisome proliferator-activated receptor-γ (PPARγ) agonists, such as rosiglitazone and pioglitazone[20, 21, 109, 110] and the dipeptidyl peptidase (DPP)-4 inhibitor, sitagliptin[18], increase EPCs levels and improve their function in diabetic patients. Regarding insulin therapy, previous *in vitro* and animal studies have demonstrated a protective role on EPCs function. In addition, more recently Marfella et al have shown that EPCs levels increased after insulin infusion for intensive glycemic control in AMI patients with hyperglycemia[111].

Besides the key effect of insulin on the metabolic control of diabetic patients, insulin also seems to improve endothelial function[112]. Several mechanisms have been proposed to explain the beneficial effect of insulin on endothelial function: reduction of free fatty acids, decrease of oxidative stress and enhancement of nitric oxide production. These mechanisms may also justify the protective role of insulin therapy on EPCs biology. However, insulin may also positively impact on EPCs by improving glucose control and removal of the adverse hyperglycemic milieu.

Despite the obvious interest in knowing the impact of chronic antidiabetic therapy on EPCs response to an AMI in diabetic patients, to date no studies have explored this issue. Therefore, it remains unclear whether antidiabetic drugs and good glycemic control of diabetic patients can completely restore chronically reduced and dysfunctional EPCs, translating into a normal neovascularization potential in response to an AMI.

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PART II

AIMS

AIMS

Endogenous EPCs form part of the body's defense against vascular damage and ischemic injury. However, as the native response of EPCs is insufficient to restore vascular homeostasis after an AMI, pharmacological stimulation of the endogenous pool of EPCs represents a promising therapeutic strategy to improve vascular repair of the ischemic myocardium. This is especially important for patients with AMI and DM, who might have reduced numbers and dysfunctional circulating EPCs.

The overall goal of this thesis was to study the potential of pharmacological modulation of the endogenous EPCs response to an AMI, focusing on the impact of statins and insulin therapy on EPCs levels and function in diabetic patients.

Therefore, the present research was divided in two phases: 1) *in vivo* quantitative study of the pool of circulating EPCs by flow cytometry, and 2) *in vitro* cell cultures for the simultaneous functional evaluation of early and late EPCs obtained from peripheral blood of AMI patients.

In the first phase of this research the specific aims were:

1. To compare the native EPCs response to an AMI between diabetics, pre-diabetics and patients with normal glucose metabolism, by quantifying circulating EPCs levels in the early phase of the AMI;

2. To evaluate the impact of glycemic control and the effect of chronic insulin therapy on circulating EPCs levels, in the acute phase of a MI;

3. To assess the impact of prior chronic statin therapy on EPCs response to an AMI;

4. To analyze the influence of the intensity of statin therapy at discharge on the evolution of circulating EPCs levels following the AMI.

In the second phase of this research, the specific aims were:

1. To functionally compare EPCs derived from diabetic with those from non-diabetic patients with AMI (cell cultures under control conditions);

2. To study the effect of hyperglycemia *per se* on EPCs function, by comparing EPCs derived from non-diabetic patients, in culture conditions mimicking clinical hyperglycemia, to those of equivalent diabetic patients when removed from the hyperglycemic milieu (cell cultures under normal glucose concentration);

3. To evaluate the ability of atorvastatin and pravastatin to reverse the deleterious effects of diabetes on EPCs function.

PART III

PUBLICATIONS

CHAPTER I

Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs

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REVIEW ARTICLE

Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs

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Abstract The role of vascular endothelium in cardiovascular disorders is well recognized. Mature endothelial cells contribute to the repair of endothelial injury, but they only have a limited capacity to do so. This has led to growing interest and further investigation into circulating endothelial progenitor cells (EPCs) and their role in vascular healing, repair, and postnatal neovascularization. The current perception of vascular health is that of a balance between ongoing injury and resultant vascular repair, mediated at least in part by circulating EPCs. Circulating EPCs play an important role in accelerating endothelialization at areas of vascular damage, and EPC enumeration is a viable strategy for assessing reparative capacity. Recent studies have shown that EPCs are affected both in number and function by several cardiovascular risk factors as well as various cardiovascular disease states, such as hypertension, hypercholesterolemia, and coronary artery disease. The present review summarizes the most relevant studies on the effects of cardiovascular drugs on vascular function and EPCs, focusing on their mechanisms of action.

Keywords Cardiovascular disease · Drugs · Endothelial dysfunction · Endothelial progenitor cells · Neovascularization

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Introduction

Endothelial dysfunction, atherosclerosis and cardiovascular disease

Atherosclerosis is a progressive and complex disease, characterized by thickening of the arterial wall, with focal complications in different vascular beds [1]. Risk factors and a genetic predisposition together seem to induce inflammatory processes that lead to cell damage and impair regeneration within the vessel wall [2]. However, despite intense efforts to determine the pathogenesis of atherosclerosis, this process remains poorly understood.

Several experimental and clinical studies have demonstrated that the endothelium, situated at the interface between blood flow and the vascular wall, plays a crucial role in the regulation of vascular tone and structure [1, 3]. For several years, the endothelial monolayer had been considered to be a simple barrier. It has become evident, however, that the endothelium is a complex organ, with

paracrine and autocrine function, which provides a “first line” physiological defense against atherosclerosis [4]. A healthy endothelium is now considered to be a major regular of vascular homeostasis, regulating the vascular tone and structure and paying a key role in angiogenesis and inflammation.

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis. Mechanisms that can contribute to endothelial dysfunction are oxidative stress, upregulation of adhesion molecules, increase in the inflammatory response and protrombotic state. Vasoactive peptides, such as angiotensin II and endothelin-1, hypercholesterolemia, hyperhomocysteinemia, and hyperglycemia may participate in these mechanisms. Apoptosis of endothelial cells can also be implicated in endothelial dysfunction [5].

Oxidative stress can inhibit the three major endothelium-dependent vasodilator pathways, i.e., nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor and seems to be a first alteration responsible for triggering endothelial dysfunction in cardiovascular diseases. The reduced bioavailability of NO, the impairment in prostanoid synthesis, including prostacyclin, thromboxane A2, and/or isoprostanes, as well as the increased release of endothelin-1 can individually or in association contribute to the initiation and progression of the impairment of endothelial function [6].

A recently discovered pathway of prostacyclin signaling involves the activation of peroxisome proliferator-activated receptors (PPARs). These receptors seem to have very important roles in the beneficial endothelial effects of prostacyclin. In fact, this endothelium-derived vasodilator seems to have angiogenic properties that are dependent on its specific action on the PPAR signaling pathway [7].

Nitric oxide is a pivotal endothelium-derived substance. It is synthesized from the substrate L-arginine via endothelial NO synthase (eNOS) and plays a crucial role in vasorelaxation, inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell migration and proliferation, and platelet aggregation [8]. A defect in NO production or its activity has been proposed as a major mechanism of endothelial dysfunction, thus contributing to atherosclerosis [9].

A growing body of evidence shows that deregulation of the endothelial NO pathway, which can cause endothelial dysfunction, is not only associated with all major cardiovascular risk factors, such as hypercholesterolemia, diabetes, hypertension, and smoking, but that it also has a profound predictive value for future atherosclerotic disease progression [10, 11]. Therefore, the dysfunctional eNOS/NO pathway is considered to be a hallmark of endothelial dysfunction [9].

Despite experimental evidence demonstrating the contribution of endothelial eNOS/NO impairment in the pathogenesis of atherosclerosis, no single underlying mechanism can fully explain endothelial dysfunction. This may simply be due to the fact that atherosclerosis is a complex disease process and that multiple regulatory mechanisms are involved in endothelial NO bioactivity.

Endothelial progenitor cells and vascular repair

Circulating endothelial progenitor cells (EPCs) derived from bone marrow were isolated for the first time in 1997 [12]. Following the publication of this initial report by Asahara et al., growing interest has focused on gaining an understanding of the mobilization, homing, and function of EPCs under several conditions and also on its potential as a new strategy in regenerative medicine.

Bone marrow-derived EPCs contribute to the re-endothelialization of injured vessels and ischemia-induced neovascularization, and they improve endothelial function [13, 14]. Therefore, circulating EPCs may represent an important endogenous repair mechanism by which the body maintains the integrity of the endothelium monolayer [12, 15, 16]. Several clinical studies have shown that the number and function of EPCs are impaired in some pathological conditions. These findings suggest that it could be very important to estimate EPCs levels and to increase their bioactivity by appropriate interventions [13].

EPCs can be mobilized into the circulation in response to angiogenic growth factors, chemokines, and cytokines released following various stimuli, such as vascular trauma [17]. Granulocyte macrophage-colony stimulating factor (GM-CSF) seems to amplify EPC mobilization, recruiting them into the site of new blood vessel formation in severely ischemic tissues [18]. Hypoxia-inducible factor-1 (HIF-1), a central transcriptional regulator of hypoxia-specific gene expression, induces the expression of signaling factors, such as stromal-derived factor (SDF-1) and vascular endothelial growth factor (VEGF), in EPCs or the endothelium and facilitates adhesion of these progenitor cells to the ischemic endothelium [19]. SDF-1, also known as CXCL12, through interaction with its receptor CXCR4 (also known as CD184), modulates angiogenesis as well as hematopoiesis [2].

Of note, EPCs are believed to exert their function by two main strategies: activating locally the endothelial cells and/or differentiating into mature endothelial cells that integrate the damaged vessels. To do this, EPCs must home to “angiogenic active” sites and then adhere to the activated/damaged endothelial cells or to the extracellular matrix, thereby contributing to the endothelial repair process [20].

Recent clinical evidence supports the concept that several cardiovascular risk factors (hypertension, diabetes, hypercholesterolemia, and smoking) as well as various

cardiovascular diseases are associated with EPC impairment, both in number and function [21]. Moreover, reduced EPC levels seem to be correlated with endothelial dysfunction and with an increased risk of cardiovascular events, compatible with the concept that impaired EPC-mediated vascular repair promotes the progression of vascular disease [22]. Importantly, it has been suggested that reduced levels of circulating EPCs represent a cellular marker that independently predicts outcome in patients with vascular disease, and the level of circulating EPCs has been proposed as a surrogate index of cumulative cardiovascular risk [2].

At the present time, EPC quantification is largely performed by two methods: (1) fluorescence-activated cell sorting (FACS) analysis of total blood cells or circulating mononuclear cells and (2) culture assay of blood-derived mononuclear cells. Circulating EPCs are characterized by the expression of CD133, CD34, and vascular endothelial growth factor receptor-2 (VEGFR2) [23]. Therefore, they can be identified and quantified based on the expression of these cell surface markers. In several studies, the marker combinations of CD34+KDR+, CD34+CD133+, and CD34+CD133+KDR+ have been the most frequently used markers for identifying human EPCs [24, 25].

It would be very interesting to develop pharmacological approaches that promote vascular repair and prevent endothelial cell apoptosis, thereby protecting the structural and functional integrity of the endothelium. Thus, within the context of promoting the health of the vascular system, it would be very beneficial to identify pharmacological interventions that could increase circulating EPCs levels and improve their bioactivity.

Stimulation of EPCs: a new putative effect of several cardiovascular drugs

Both experimental and human studies have demonstrated that the number of functionally active EPCs is regulated not only by various angiogenic cytokines and cardiovascular risk factors, but also by some interventions, including lifestyle modification (aerobic exercise, body weight loss, and smoking cessation) and by pharmacological intervention [15, 26].

EPCs exist in very small numbers, especially in the circulating blood of adults, where they only account for approximately 0.01% of all cells [17].

Several experimental and even clinical studies have demonstrated the impact of various pharmacological agents on the number and function of EPCs (Table 1). This impact could be of therapeutic relevance because persistent stimulation of EPCs by targeted pharmacological intervention could, at least theoretically, repair endothelial injury and prevent progression of atherosclerotic vascular disease in patients at risk.

In this review we summarize recent data on the effects of cardiovascular drugs on mobilization and functional activity of EPCs, with an attempt to focus on the mechanisms of action involved.

Table 1 Cardiovascular drugs with potential positive effects on EPCs

Cardiovascular drugs	Effects on EPCs and potential mechanisms of action
Lipid-lowering drugs	
Statins rosuvastatin simvastatin atorvastatin fluvastatin cerivastatin	Improvement in proliferation, differentiation, migratory function of EPCs (important role of eNOS) Delay of EPCs senescence (via the Akt signaling pathway) Increasing of EPC homing (through the upregulation of endothelial integrin subunits $\alpha5$, $\beta1$, α_v and $\beta5$)
Renin–angiotensin-aldosterone system active agents	
Spironolactone	Attenuation of the inhibitory effect of aldosterone on EPC formation by aldosterone (via the Akt signaling pathway)
ACE-Inhibitors enalapril ramipril perindopril quinapril	Improvement in EPC mobilization (by increasing circulating SDF-1α and reducing SDF-1α in the bone marrow)
Angiotensin II receptor blockers olmesartan irbesartan losartan, candesartan telmisartan	Increase in number of circulating EPCs (by an antioxidant effect)
Antianginals	
Dihydropyridine calcium channel blockers benidipine nisoldipine nifedipine	Induction of EPC mobilization and increase in number of circulating EPCs (via the PI3K/Akt signaling pathway and the antioxidant system)
Beta blockers celiprolol	Increase in the number of circulating EPCs (via inhibition of oxidative stress in EPCs)
Nitrates nitroglycerine	Increase in level of circulating EPCs (by release of NO)
Aspirin (in a low dose)	Promotion of EPC migration and adhesion (?) Delay of EPC senescence (?)

EPCs Endothelial progenitor cells; eNOS, endothelial nitric oxide synthase; ACE-inhibitors, angiotensin-converting enzyme inhibitors; SDF, stromal-derived factor; PI3K, phosphatidylinositol 3-kinase; NO, nitric oxide

Statins

Endothelial dysfunction is frequently seen in hypercholesterolemic patients [3]. Unfortunately, a growing body of evidence indicates that hypercholesterolemia not only exerts a direct harmful effect on the endothelium but also indirectly reduces both the availability and functionality of EPCs, thus limiting EPC-mediated vascular repair [27].

Low-density lipoproteins (LDLs) play an active role in the onset and progression of atherosclerosis [28]. The impairment of endothelium-dependent vascular relaxation secondary to a decrease in NO bioavailability is one of the early deleterious effects produced by high plasma levels of LDLs [29]. It has also been shown that chronic hypercholesterolemia favors a proapoptotic status of vascular endothelial cells. The mechanism responsible for this effect has been extensively analyzed and seems to be mediated by reactive oxygen species (ROS) [30, 31].

The effectiveness of different 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) to reduce cholesterol levels and prevent cardiovascular morbidity and mortality has been consistently demonstrated [29]. A more recent development has been the demonstration that statins also exert benefits not related to the reduction of cholesterol levels; these are known as pleiotropic effects [32]. In addition to their lipid-lowering property, statins are also able to reduce vascular inflammation [33], decrease platelet aggregation and thrombus deposition [34], and increase endothelium-derived NO production [35]. Thus, although statins were initially designed to lower LDL-cholesterol levels, they have become established drugs in the treatment of coronary artery disease (CAD) even in patients with normal LDL levels, due to their pleiotropic effects [32]. In fact, increasing evidence supports profound, positive effects of statins on endothelial cell function through both cholesterol-dependent and cholesterol-independent effects [36, 37].

It is widely recognized that statins increase NO production by several mechanisms, including the upregulation of eNOS mRNA and protein levels and the restoration of eNOS activity reduced by oxLDLs or atherogenic native LDLs [38, 39]. Moreover, recent studies have shown that statins also exert a beneficial effect on angiogenesis and EPC bioactivity, both mechanisms that could also be associated with the significant improvement of endothelial function produced by these drugs [25, 29, 40, 41]. Therefore, augmentation of circulating EPCs by statin therapy may significantly contribute to the stimulation of neovascularization that follows tissue ischemia.

Previous data suggest that the modulation of EPC kinetics after statin treatment is unrelated to the decrease in serum LDL-cholesterol levels [25]. Landemesser et al. have demonstrated that similar reductions in LDL-cholesterol with simvastatin and ezetimibe in patients with chronic heart failure resulted in different effects on endothelial function and EPCs. There was a marked increase in functionally active EPCs after 4 weeks of statin treatment, whereas ezetimibe therapy had no effect on EPCs [42]. However, the exact mechanisms behind these serum lipid-independent effects of statins on EPCs kinetics are still not well understood.

Statin therapy and angiogenesis: mechanisms of action

Several clinical studies have clearly shown that statin therapy improves angiogenesis and EPC bioactivity. Here, we present the most relevant data on these effects and discuss the potential intracellular mechanisms involved.

Accumulating evidence suggests that several statins (rosuvastatin [43], simvastatin [42, 44, 45], atorvastatin [25, 46–48], fluvastatin [49], and cerivastatin [50]) mobilize EPCs, induce proliferation and differentiation, improve migratory function, have anti-apoptotic effects over EPCs, and increase EPC homing. However, studies with pravastatin have suggested that this statin is not as efficient as others in improving the number and function of EPCs [46, 51]. This observation, however, should be further investigated.

Several experiments establish a novel role for statins, analogous to that described for EPC-modulating cytokines, in the regulation of postnatal neovascularization [25, 41]. Statins seem to be a major stimuli for bone marrow-derived EPCs **mobilization**, thus improving vasculogenesis and re-endothelization [25, 41]. This effect on EPC activity may represent a novel pleiotropic effect of statin therapy [52].

There is increasing evidence for statin treatment increasing the number of circulating EPCs, based on FACS detection of CD34+/KDR+ cells or as assessed in vitro by culture assays of early EPCs. Vasa et al. showed that atorvastatin increased the number of circulating EPCs in patients with stable CAD. Moreover, this effect was as early as 1 week after the initiation of atorvastatin therapy and had risen to approximately threefold at 3–4 weeks after its introduction [25].

Dimmeler et al. demonstrated that the increase in the number of EPCs by atorvastatin is at least equipotent as the prototypic angiogenic cytokine VEGF, which is known to promote EPC mobilization and **differentiation** [40]. These researchers concluded that it was unlikely that the effects of statins were mediated via the upregulation of angiogenic growth factors. It would appear that statins augment circulating levels of bone marrow hematopoietic precursor cells and directly induce the differentiation of these precursor cells into EPCs. Thus, statins may influence not only the mobilization of hematopoietic progenitor cells, as has been shown for VEGF, but they may also modulate differentiation [40].

The effects of statins on eNOS appear to be critical for EPC mobilization and **proliferation**. Landmesser et al. showed a marked improvement in EPC mobilization and myocardial neovascularization after atorvastatin treatment in wild-type mice, while it had no effect in eNOS-deficient mice (eNOS−/−) [47]. Furthermore, in wild-type animals treated with cerivastatin, the inhibition of eNOS by nitro-L-arginine-methyl ester prevented the formation of angiotubes and collateral growth in response to ischemia, suggesting that eNOS is essential for statin to be able to enhance blood flow recovery after ischemia [50, 53].

Accumulating evidence suggests that EPCs treated with statins not only show an increased ability to form colonies and to improve proliferation [41, 54], but also demonstrate enhanced **migratory** capacity [25, 41, 48].

With respect to the statin-mediated delay of EPC **senescence**, the upregulation of the telomere-capping protein telomere repeat-binding factor TRF2 in EPCs (that prevents telomerase dysfunction) rather than an increase in telomere length is now thought to explain how statins favorably affect senescence in human EPCs [48, 54]. Human telomeres are bound by two double-strand telomere binding proteins, TRF1 and TRF2. The removal of TRF2 triggers apoptosis or senescence [55]. Premature senescence of cultured cells can be triggered by oxidative stress, leading to telomere dysfunction [56, 57]. Recent experimental and clinical evidence supports the possibility that telomere dysfunction can occur because of the loss of TRF2, implying a role of telomere "uncapping" rather than telomere shortening as an explanation for senescence [58, 59]. Spyridopoulos et al. have demonstrated that the delay of EPC premature senescence produced by statins is independent of mean telomere length [48]. In this study, exogenous TRF2 reproduced the statin effect, and a dominant-interfering mutation blocked this senescence delay, implicating TRF2 in this process [48]. The beneficial effects of statins on telomere function have also been related to ability of statins to enhance the migratory capacity of EPCs. It is possible that the induction of TRF2 by statins leads to "juvenation" of EPCs because replicative senescence is delayed. Therefore, these younger cells would migrate faster than older cells primarily due to their age characteristics [48].

There is emerging evidence suggesting that the phosphatidylinositol 3-kinase and Akt-dependent signaling pathway (PI3K/Akt) plays a critical role in statin-induced neovascularization and improvement in EPC viability [40]. In mature endothelial cells, it has been shown that the kinase Akt acts downstream of the angiogenic growth factors VEGF and angiopoietin, promoting endothelial cell survival [60]. The activation of Akt leads to the inhibition of endothelial cell apoptosis, stimulates endothelial NO synthesis, and mediates VEGF-induced endothelial cell migration [60, 61]. Akt has also been shown to be essential for EPC migration induced by VEGF and for the differentiation of EPCs [61]. Moreover, recent studies have identified Akt as a target for statins in their role as a modifier of EPC kinetics [40, 44]. It seems that statins rapidly activate Akt signaling in EPCs, enhance phosphorylation of the endogenous Akt substrate eNOS, inhibit apoptosis, and accelerate the formation of vascular structures in vitro in an Akt-dependent manner [41, 44]. Of note, the promotion of EPC proliferation, migration, and cell survival in vitro via the Akt signaling pathway by statins has been confirmed by functional blocking with dominant-negative Akt overexpression [41].

Finally, the increased adhesiveness towards endothelial cells reported in statin-treated EPCs, which could increase EPC **homing** at sites of vascular injury, seems to rely on the upregulation of endothelial integrin subunits α5, β1, α_v and β5 [45].

In conclusion, multiple studies support that the improvement of EPC functions and neovascularization promoted by statins could contribute to the clinical benefit of these drugs and that these effects are—at least in part—independent of their lipid-lowering properties. However, several questions remain unanswered and warrant further investigation: Which mechanisms are involved in the release of EPCs by statin treatment? Is this release caused by enhanced proliferation and differentiation of committed stem cells within the bone marrow, or is it due to an increased release of a preexisting pool? What are the exact mechanisms governing these events? What makes endothelial cells attach to the injured vessel wall? How is this modulated by statins?

In fact, despite intense efforts to determine the exact mechanisms whereby statins improve EPCs function, their mechanism of action remains poorly understood.

Renin–angiotensin–aldosterone system active agents

The renin–angiotensin system (RAS) [41] is a circulating hormonal system that regulates blood pressure and flow. It is recognized that angiotensin II (Ang II) contributes to the development of atherosclerosis and promotes cardiovascular remodeling through its induction of cardiac hypertrophy, inflammation, apoptosis, and fibrosis through its action on the angiotensin type 1 receptor (AT1) [62]. Furthermore, AT1-receptor activation causes superoxide radical release via the NAPDH oxidase pathway, thereby increasing oxidative stress in the vascular wall, which leads to endothelial dysfunction [62, 63]. In contrast, angiotensin type 2 receptors (AT2) promote vasodilatation and are responsible for antifibrotic, anti-apoptotic, and anti-inflammatory effects [64].

AT2 receptors are highly expressed in the developing fetus, but they decline rapidly after birth. In the adult, the

AT2-receptor is predominantly expressed in the brain and adrenals, being expressed in lower levels elsewhere. Therefore, angiotensin-dependent functions are primarily the result of AT1-receptor activation and, to a lesser extent, AT2-receptor activation [63]. Further research to assess the potential clinical benefits of specifically targeting the AT2-receptor could be of great value.

Ang II is formed by the activation of a cascade of enzymatic conversions from angiotensin I by angiotensin converting enzyme (ACE). Alternative proteases are present in the human heart (e.g., chymase), which produce Ang II independently of ACE [65]. ACE-inhibitors reduce the formation of Ang II and lead to the accumulation of NO and bradykinin [64]. The reduced stimulation of both AT1- and AT2-receptors is partially offset by an increase in AT1-receptor stimulation of the Ang II formed by the alternative pathway, potentially contributing to the "angiotensin escape phenomenon" observed in patients on chronic ACE-inhibitor treatment [64].

Recent evidence suggests that other angiotensin peptide fragments, including Ang-(1–7) and Ang-(3–8) (known as Ang IV), may bind to non-AT1 receptor subtypes [e.g., AT2, AT4, and Ang (1–7)/*mas* receptor] to counter-regulate the effects mediated via the AT1. Ang-(1–7) is formed directly from Ang I via the action of several tissue-specific endopeptidases or from Ang II via ACE. It interacts with the G-protein-coupled receptor *mas* and opposes the actions of Ang II by stimulating the release of prostacyclin and NO [67].

The Ang II metabolite, Ang IV, preferentially binds to a novel angiotensin binding site, which shows a poor affinity for Ang II (the AT4-receptor). A growing list of physiological functions has been attributed to the AT4-receptor, including an increase of blood flow in various vascular beds, antagonism of Ang II-induced hypertrophic changes, and modulation of diastolic function. Moreover, Ang IV has been shown to reverse endothelial dysfunction [66].

Both ACE-inhibitors and angiotensin II receptor blockers (ARBs), by increasing Ang I availability, elevate Ang-(1–7), which may contribute to the antihypertensive effects of these drugs.

Blockade of the RAS would be expected to improve endothelial function. There is a strong rationale for blockade of the RAS early in the cardio–reno–vascular continuum in order to prevent adverse events in patients with cardio-vascular disease.

Murohara et al. have reported that the Ang II–AT1-receptor pathway plays important roles in angiogenesis associated with ischemia and tumor [68, 69]. Moreover, Imanishi et al. have demonstrated that Ang II accelerates EPC senescence by a gp91phox-mediated increase of oxidative stress, resulting in EPCs dysfunction [70].

Spironolactone

In addition to its negative effects attributable to elevation of the blood pressure, aldosterone has been suggested to contribute to vascular damage by directly acting on the vasculature. Moreover, aldosterone can cause swelling of the endothelial cells and induce proinflammatory molecules in peripheral blood mononuclear cells [71, 72]. More recently, it has been demonstrated that aldosterone inhibits the formation of bone marrow-derived progenitor cells, at least partly, by attenuating VEGFR-2 expression and subsequent Akt signaling and that this effect of aldosterone was attenuated by cotreatment with spironolactone [73].

ACE-inhibitors

Although modulation of the RAS, by inhibition of ACE or through blockage of Ang II type 1 receptors, reduces mortality rates and major non-fatal cardiovascular events in high-risk patients, [74], the underlying mechanisms behind these beneficial effects have not been fully elucidated. There is increasing evidence suggesting that one of the pleiotropic effects of ACE-inhibitors on the cardiovascular system involves modulation of the number and functional activity of EPCs [75]. Moreover, this seems to be a class effect since an improvement in EPC biology has been demonstrated for several ACE-inhibitors (enalapril, ramipril, perindopril, and quinapril).

Treatment with enalapril has shown to benefit neovascularization in a murine hindlimb ischemia model. This ACE-inhibitor seems to augment circulating EPC levels and appears to influence EPCs through stimulating mobilization rather than maturation. In response to ischemic stress, enalapril, transiently and significantly increased the concentration of circulating SDF-1α but reduced its concentration in the bone marrow, suggesting that reduced binding of EPCs to SDF-1α in bone marrow may contribute to the release and mobilization of EPCs after ACE-inhibitor therapy [75]. The concentration gradient of plasma SDF-1α between the bone marrow and the peripheral blood may, at least in part, result from increased bone marrow activation of dipeptidylpeptidase IV (DPP IV; CD26), a cell surface endopeptidase that cleaves chemokines, such as SDF-1α [75]. Thus, the beneficial effects of enalapril seem to be at least in part due to its ability to alter the CD26/ DPP IV system, and SDF-1α seems to be critical in the mechanism through which enalapril modulates EPC mobilization [75].

Likewise, Min et al. have demonstrated that treatment with ramipril for 4 weeks increased circulating EPCs levels in patients with stable CAD [76]. Moreover, the increased number of EPCs was paralleled by an enhancement of the migratory, proliferative, adhesive, and in vitro vasculogenesis capacity of isolated EPCs [76]. In this work, the

investigators suggested that the modulation of EPC kinetics after the ramipril treatment was unrelated to the decrease in blood pressure levels [76]. Moreover, as they observed that ramipril increased NO levels, they speculated that the activation of the bradykinin B2-receptor pathway by ACE-inhibitors may contribute to the observed effects of ramipril on the functional improvement of EPCs [76].

Another study demonstrated that the addition of quinapril and metoprolol to EPC therapy induced neovascularization and reduced the number of apoptotic cardiomyocytes. However, the mechanisms by which this positive effect occurred remain unclear [77].

Finally, perindopril is a long-acting ACE-inhibitor that has been demonstrated to be able to increase the number of circulating EPCs and to re-establish the ability of bone marrow mononuclear cells to differentiate into EPCs in a hindlimb ischemia model in spontaneously hypertensive rats, either alone or in combination with indapamide [78].

In conclusion, there is mounting evidence that ACE inhibition improves the biology of EPCs independently of a vasodilator or hemodynamic effect. However, again, many questions remain unsolved regarding the angiogenic effects of these widely prescribed cardiovascular drugs: What is the exact role of NO in ACE-inhibition-dependent vasculogenesis? Are all ACE-inhibitors equivalent regarding their angiogenic action? Would the magnitude of EPC improvement be similar in diabetic patients? What about other conditions? Would ACE-inhibitors be able to improve EPCs biology in patients with metabolic syndrome or heart failure?

Angiotensin II receptor blockers

Blockade of AT1 receptors results in the inhibition of a variety of deleterious effects. Furthermore, as circulating Ang II increases during treatment with AT1 blockers, the selective blockade of AT1 would be expected to result in the stimulation of unblocked AT2-receptors, thereby enhancing AT2-mediated vasodilatation and antiproliferative effects.

ARBs (olmesartan, irbesartan, losartan, candesartan, telmisartan) have consistently been reported to increase the number of EPCs [26, 79–82].

Bahlmann et al. demonstrated that Ang II subtype 1-receptor blockage (with olmesartan or irbersartan) increased the number of EPCs in patients with diabetes above that found in healthy subjects and that this effect was independent from their blood pressure-lowering action [26]. Similarly, Chen et al. demonstrated that the combined treatment with olmesartan and pravastatin increased the number of circulating EPCs in a murine model of balloon injury of the carotid arteries [79]. Additionally, studies in spontaneously hypertensive rats have shown that losartan and candesartan markedly increased the number and colony formation of EPCs and exerted a favorable effect on EPC migration, independently of their effects on blood pressure. The authors of these published studies suggested that the positive effects on EPC number and function are in part due to an antioxidant effect [81, 82].

Similarly as for statins, the so-called pleiotropic effects of ARBs are attracting the increasing interest in the cardiovascular community, including their antidiabetic and anti-inflammatory action [83, 84].

Some ARBs have also been shown to be selective PPAR-γ modulators, implicating an involvement in the metabolism, proliferation, and inflammation of cardiovascular cells [85]. Telmisartan may be especially effective in slowing down disease processes of the metabolic syndrome on account of its effect on PPAR-γ [85]. It has recently been demonstrated that telmisartan has a more potent anti-inflammatory effect than ramipril after an acute coronary syndrome. However, there are no differences between the two drugs in terms of the magnitude of improvement on EPC mobilization [80].

At the present time it is unknown whether the upregulation of progenitor cells by ARBs is linked mechanistically to the impact of these pharmacologic agents on endothelial function or whether it is just a parallel effect. Therapy with ARBs may provide a novel and effective therapeutic strategy for the repair of vascular injury. However, intracellular mechanisms involved in EPC stimulation by ARBs have yet to be explored. Further studies are required to determine the precise mechanisms of ARBs in modifying the number and function of EPCs.

Antianginals: ß-blockers, calcium antagonists, and nitrates

Multiple experimental models and a number of randomized, placebo-controlled, double-blind clinical trials have suggested anti-atherosclerosis effects for all of these antianginal agents [86]. How these effects are related to modification of EPC biology or to the documented reduction in adverse outcomes in patients with myocardial infarction, heart failure, or hypertension with ß-blockers is not clear.

Calcium antagonists: dihydropyridine calcium channel blockers

Most calcium antagonists have antioxidant effects and reduce experimental inflammatory cell invasion. Dihydropyridine-type calcium antagonists increase NO, block lipid peroxidation, and may be associated with a reduction in cardiovascular events [87].

Evidence that dihydropyridine calcium channel blockers affect the functional activity of EPCs was initially obtained

with benidipine in culture studies. Treatment of murine mononuclear cells with this dihydropyridine calcium channel blocker was shown to increase the number of early EPCs after 7 days in culture, probably via the PI3K/Akt signaling pathway [88]. Additionally, nisoldipine, another L-type Ca^{2+} channel blocker, seems to induce the mobilization of EPCs in patients with essential hypertension independently of the blood pressure-lowering effect [89].

More recently, it has been demonstrated that nifedipine also improves the functional capacity of EPCs. This dihydropyridine calcium channel blocker enhances VEGF release from EPCs and improves the migratory capacity of cultured EPCs [90]. This study showed a nifedipine-dependent upregulation of manganese superoxide dismutase, which is known to confer resistance to oxidants, suggesting a role of the antioxidant system for the effects of nifedipine on EPCs [90].

It should be noted, however, that the majority of favorable results with calcium antagonists have been obtained from in vitro studies and that these need to be confirmed clinically. In addition, one could question whether these effects will persist in the context of cardiovascular disease, such as acute coronary syndromes and heart failure.

Beta blockers

It has recently been demonstrated that another group of cardiovascular drugs improves EPCs number and function—the beta blockers.

In spontaneously hypertensive rats, celiprolol has been shown to increase the number of circulating EPCs and to stimulate EPC colony formation and migration, while decreasing EPC senescence. This beta blocker inhibited oxidative stress in EPCs, suggesting that its beneficial effects in EPCs are mediated by its antioxidative properties [91].

Further studies with other beta blockers are required to determine if these benefits in EPC biology are exclusive of celiprolol or a class effect of all beta blockers.

Nitrates

Nitrate compounds have been used in the treatment of myocardial ischemia for more than 100 years. Their common mechanism is the release of NO, which is a major regulator of EPC mobilization, differentiation, and function [92]. Nevertheless, the majority of nitrates additionally stimulate the production of ROS, a process that is partly involved in the development of nitrate tolerance and may counteract the beneficial effects of NO on the endothelium [93].

In a small clinical trial, nitroglycerine treatment of healthy volunteers increased the level of circulating CD34 progenitor cells but also enhanced the susceptibility of expanded EPCs to apoptosis [94]. Moreover, ex vivo nitroglycerine exposure attenuated EPC differentiation, function, and survival [94].

More recently, Thum et al. demonstrated that different nitrates have a number of important effects on the circulating levels and function of EPCs [95]. Therapy with two different long-acting nitrates (nitroglycerine and isosorbide-5-dinitrate) increased the levels of circulating EPCs. However, functionally, there were strong differences between the tested nitrates, with isosorbide-5-dinitrate increasing ROS formation and impairing EPC function and nitroglycerine having favorable effects even on EPC function [95].

In one study, a considerable increase in oxidative stress was observed after treatment with certain long-acting nitrates, such as isosorbide-5-dinitrate [96]. In contrast, treatment with nitroglycerine did not cause tolerance and was not associated with evidence of increased ROS levels [95, 96]. Thum et al. concluded that the differences between the two studied long-acting nitrates in terms of induction of oxidative stress in EPCs may explain their differences in EPC biology [95].

In conclusion, further prospective studies are needed that determine the long-term effects of organic nitrates on number and function of EPCs.

Antiplatelet therapy: aspirin

Aspirin is widely used in the primary and secondary prevention of vascular disease. It is a cornerstone of therapy in acute coronary syndromes and has been shown to reduce atherosclerosis-related events in a multitude of clinical studies [97, 98]. Kharbanda et al. have reported a protective effect of aspirin against endothelial dysfunction [99].

Surprisingly, in a study with healthy humans, Chen et al. concluded that aspirin decreased the number of EPCs, decreased the proliferative, migratory, adhesive, and in vitro vasculogenesis capacity of EPCs, and decreased EPC production of iNOS in a concentration- and time-dependent manner [100]. However, recent in vivo observations have suggested that low-dose aspirin promotes the migration and adhesion and delays the onset of senescence of EPCs [101].

These studies seem to be contradictory as they provide evidence for both an inhibitory and a stimulating effect of aspirin on EPC biology. Therefore, the effects of aspirin on EPCs must be studied further.

Conclusion

Given the fact that EPCs contribute to postnatal neovascularization, an improved understanding of the regula-

tion of EPCs could lead to new insights into the pathogenesis of vasculogenesis. Several cardiovascular drugs, such as statins, ACE-inhibitors, ARBs, and beta blockers have demonstrated a direct benefit on circulating EPCs that may contribute to their overall effects on the vascular wall. The augmentation of the number of EPCs through pharmacological modulation of the signaling pathways may be a novel strategy to improve neovascularization after ischemia and, thereby, provide a therapeutic concept for improving the numbers of EPCs in patients with cardiovascular diseases.

In conclusion, a more complete understanding of the complex factors regulating EPC biology is required in order to develop and investigate more focused clinical therapies.

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CHAPTER II

Challenges in vascular repair by endothelial progenitor cells in diabetic patients

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Challenges in Vascular Repair by Endothelial Progenitor Cells in Diabetic Patients

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Abstract: Endothelial progenitor cells (EPCs) are a special type of stem cells, derived from bone marrow that can be mobilized to the peripheral circulation in response to many stimuli. EPCs play a crucial role in the vascular repair, as well as in neovascularization processes. Recent studies have shown that EPCs are impaired, both in number and function, in diabetic patients independently of other cardiovascular risk factors.

Accelerated atherosclerosis is probably the most devastating among diabetes complications and endothelial dysfunction might be the beginning of the atherosclerosis. The impairment of EPCs seems to significantly contribute to atherogenesis and atherosclerotic disease progression in diabetes.

Autologous EPCs therapy is a promising treatment option for vascular complications requiring therapeutic revascularization and vascular repair. Diabetic patients represent a population that may benefit from cell-based therapy; however, the dysfunction of their endogenous cells may limit the feasibility of this approach. In fact, EPCs isolated from these patients for autologous cell transplantation may retain their dysfunctional characteristics *in vivo* and as a consequence display a reduced capacity to improve therapeutic neovascularization.

In the present review, we summarize the most relevant mechanisms underlying EPC dysfunction in diabetes.

Keywords: Endothelial progenitor cells, diabetes, neovascularization, endothelial dysfunction, hyperglycemia, insulin resistance.

INTRODUCTION

Diabetes, Endothelial Dysfunction and Atherosclerosis

Diabetes is a common, chronic disease which has already reached epidemic proportions in the Western world [1]. Moreover, it is likely that the incidence of type 2 diabetes will rise as a consequence of lifestyle patterns contributing to obesity [2].

Accelerated atherosclerosis is probably the most devastating among diabetes complications and endothelial dysfunction might be the beginning of the atherosclerosis. Diabetes mellitus (DM) is characterized by early endothelial dysfunction that seems to be involved in the development of macro and microvascular complications of diabetic vascular diseases and in the subsequent increased risk of cardiovascular events and mortality [2, 3].

Endothelial dysfunction in type 2 diabetic patients seems to be related to an increased oxidative stress, and inflammation [4]. Nitric oxide (NO), produced from L-arginine by endothelial NO synthase (eNOS), is a pivotal endothelium-derived substance which possesses vasodilatory, anti-inflammatory, antiplatelet and antioxidant properties, representing a key marker in vascular health. NO is rapidly inactivated in the vessel wall by reactive oxygen species (ROS). The relative production by endothelial cells of NO and ROS is, therefore, of critical importance in vascular homoeostasis [5].

Decreased NO bioavailability has been proposed as one of the determinants of vascular damage in diabetes which is itself an independent predictor of future cardiovascular events [6].

Under physiological conditions, eNOS confers anti-arteriosclerotic vascular protection [7]. In general, increased eNOS expression is considered to be beneficial. However, under certain pathophysiological conditions, upregulation of eNOS expression is associated with reduced endothelium-dependent vasodilatation explained by the so-called “eNOS uncoupling” [8]. Under this condition eNOS itself can be a source of $O2^{-}$, instead of NO [8]. Diabetes-associated eNOS uncoupling results in decreased NO bioavailibility, increased superoxide production, and disrupted eNOS dimer formation within the vascular wall [9].

Recent research reveals a potential role of "eNOS uncoupling" in endothelial dysfunction in atherosclerosis. Moreover, this mechanism also seems important for endothelial dysfunction associated with diabetes mellitus [6].

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Endothelial Repair Mediated by Endothelial Progenitor Cells

Regeneration and reconstruction of the vascular endothelium is essential in vascular repair processes. Mature local adjacent endothelial cells are terminally differentiated cells with low proliferative potential and a limited capacity to repair damaged vessels [10]. In 1997, Asahara *et al*. described for the first time a population of putative endothelial progenitor cells (EPCs) in human peripheral blood [11]. Since then, numerous studies have suggested that EPCs promote endothelial repair after vascular injury.

EPCs are circulating cells that originate from the bone marrow, and contribute to re-endothelialization of injured vessels as well as ischemia-induced neovascularization through their capacity to proliferate, migrate, differentiate and as a source of paracrine factors for pro-angiogenic cytokines [12]. These EPCs co-express surface markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (CD146 and VEGFR2, also known as KDR) [13, 14].

The recruitment of EPCs from bone marrow to homing sites of angiogenesis or blood vessel regeneration occurs in response to growth factors and cytokines including vascular endothelial growth factor (VEGF), stromal-cell-derived factor-1α (SDF-1α), erythropoietin and oestrogens [15]. Vascular injury, particularly when associated with tissue ischaemia, is the strongest stimulus for EPCs mobilization, acting through the release of cytokines and chemokines in response to hypoxia [16].

Expression and phosphorylation of eNOS are known to be essential for the survival, migration, and angiogenesis of either EPCs or endothelial cells. NO derived from eNOS has been identified as promoting the mobilization of EPCs from the bone marrow through nitrosylation and elevated VEGF expression. NO bioavailability is essential for normal functional activity of EPCs [17].

EPCs are believed to exert their function either through direct differentiation in mature endothelial cells that integrate the damaged vessels, or by functioning as perivascular support cells that produce additional factors that stimulate repair [18, 19].

Circulating EPCs may represent an important endogenous repair mechanism to maintain the normal endothelial function.

POTENTIAL MECHANISMS UNDERLYING EPC DYSFUNCTION IN DIABETES

Endothelial Progenitor Cells in Diabetes

Today it has become clear that there is an inverse relation between the number and function of EPCs and several traditional risk factors. The metabolic syndrome, hypertension, family history of coronary artery disease, hypercholesterolemia and smoking are associated with EPCs impairment, both in number and function [20]. Likewise, recent reports showed reduced numbers and impaired function of EPCs in diabetic patients that are observed independently of other cardiovascular risk factors [21]. Moreover, emerging evidence suggests that there is a negative correlation between the severity of diabetes and EPC count and function [4]. Table **1** summarizes human studies which have investigated the effects of diabetes on EPCs.

Of note, reduced levels of circulating EPCs are now considered as an independent marker of poor outcome in patients with vascular disease and the level of circulating EPCs has been proposed as a surrogate index of cumulative cardiovascular risk [22].

The mechanisms contributing to the reduction of circulating EPCs in diabetic patients are presently not fully understood, however, it may reflect a shortened peripheral survival of these cells, and/or a poor mobilization of EPCs from bone marrow [23].

EPC dysfunction in type 2 DM is linked to oxidative stress [24]. Decreased NO bioavailability seems to be involved in impaired EPC mobilization from bone marrow to peripheral blood [25].

Impact of Hyperglycemia on EPC Bioavailability

It is well known that hyperglycemia impairs vascular endothelial function, and contributes to the vasculopathies of diabetes mellitus, even with tight glycemic control [26].

In a recent study of 36 type 2 diabetic patients, hyperglycemia significantly decreased EPC viability and proliferation, and increased apoptosis, in a dose-dependent manner.

Experimental evidence supports the notion that hyperglycemia decreases endothelium-derived NO [2]. Prolonged exposure to hyperglycemia in type 2 diabetes leads to excessive O2- generation which, in turn, negatively affects the ability of EPCs to repair the vascular endothelium [23]. Glucose stress in EPCs can generate O2- *via* several processes that include glucose auto-oxidation, increased protein kinase C and NAD(P)H oxidase activity. Importantly, it is likely that hyperglycemia inhibits the Phosphatidylinositol 3'-Kinase (PI3k)/serine-threonine kinase (Akt)/eNOS pathway in EPCs, thus resulting in reduced viability and proliferation [23].

Recently, Urbich *et al.* have demonstrated that hyperglycemia significantly reduces the protein expression and activity of cathepsin L, which is involved in matrix degradation and required for invasion of EPC into the ischemic tissue, and, thereby, may limit the functional capacity of EPC to improve neovascularization in diabetics [27].

EPC numbers are significantly higher in diabetic patients with good glycemic control compared with those with poor glycemic control, supporting the hypothesis that EPC function can be improved by strict control of blood glucose, and thereby preventing or ameliorating the development of severe vascular complications in diabetics [23]. However, EPC levels in patients with good glycemic control did not reach levels of healthy controls. In fact, there appears to be some

Table 1 - Summary of Human Studies Investigating EPCs in Diabetes

Study Type	Principal Findings	Year	References
Cell culture from patients with type 2 diabetes	Diabetes was associated with impaired EPC proliferation, adhesion to endothelial monolayers and incorporation into endothelial tubular capillary-like networks	2002	[39]
	Rosiglitazone increased EPC numbers and migratory function	2004	[40]
Transplantation of PBMNCs in type 2 diabetic patients	Autologous transplantation of G-CSF mobilized peripheral blood mononuclear cells improved critical limb ischemia in diabetes	2005	[41]
FACS enumeration of EPCs from patients with type 2 diabetes	Diabetes was associated with reduced circulating EPCs in patients with peripheral arterial disease and EPC levels correlated inversely with plasma glucose.	2005	[42]
Cell culture from patients with type 2 diabetes	Pioglitazone added to metformin increased EPC proliferation and colony formation, and attenuated EPC apoptosis in diabetic patients	2006	[43]
	In type 2 diabetic patients, reendothelialization capacity of EPCs was severely impaired at least partially as a result of reduced NO bioavailability.	2007	[44]
	Uncoupling of the eNOS resulting in O2- formation instead of NO impaired EPC mobilization and function in diabetic patients	2007	[45]
	Insulin-mediated activation of the IGF-1 receptor lead to an increased clonogenic and angiogenic potential of EPCs	2008	[46]
FACS enumeration of EPCs from patients with type 2 diabetes	Daily thiamine intake was positively correlated with the circulating number of EPCs in patients with type 2 diabetes	2008	[47]
	Generalised decrease of putative endothelial progenitors and CXCR4-positive peripheral blood was observed in type 2 diabetic patients. This reduction was negatively associated with disease severity.	2008	[48]
Cell culture from patients with type 2 diabetes	Nitric oxide and superoxide dismutase modulate endothelial progenitor cell function in type 2 diabetes mellitus	2009	[4]
	Clopidogrel increased the expression of EPC phosphorylated Akt and phosphorylated adenosine monophosphate kinase in the peripheral blood of patients with type 2 diabetes mellitus	2010	[49]

G-CSF, granulocyte colony-stimulating factor; PBMNCs, peripheral blood mononuclear cells; EPC, endothelial progenitor cells; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; IGF-1, Insulin-like growth factor 1

degree of endothelial damage which is irreversible, even after strict glycemic control [23, 28].

Insulin Resistance, Free Fatty Acid Liberation and EPCs

Insulin resistance is a key feature of obesity, the metabolic syndrome, pre-diabetes and type 2 diabetes which promotes the development of atherosclerosis [2]. Insulin resistance is associated with impaired downstream signal transduction when insulin binds to its receptor, so reducing glucose uptake in metabolic tissues. Endothelial dysfunction, dyslipidaemia, inflammation and a pro-thrombotic state are all hallmarks of insulin-resistant states [16].

Insulin increases the activity of NOS *via* activation of PI3k and Akt kinase stimulating NO production in endothelial cells. In insulin-resistant subjects, NO-dependent vasodilation is reduced due to impairment in insulin signal transduction *via* the PI3k/Akt pathway, and lower ability of insulin to activate NOS and produce NO [29].

As a result of the abnormalities in NO bioavailability and PI3K/Akt signalling, both of which play a crucial role in EPC mobilization from the bone marrow, insulin resistance is closely associated with reduced circulating EPCs [30].

Additionally, insulin resistance is associated with elevations in free fatty acid levels. In fact, free fatty acids are one important link between insulin resistance, and type 2 diabetes. Circulating levels of free fatty acids are elevated in diabetes because of their excess liberation from adipose tissue and diminished uptake by skeletal muscle [2].

Free fatty acids may impair endothelial function through several mechanisms, including increased production of oxygen-derived free radicals, activation of protein kinase C, and exacerbation of dyslipidemia. Hyperglycemia and free fatty acids may stimulate ROS production through protein kinase C-dependent activation of NAD(P)H oxidase [31].

Recently, Guo *et al*. have demonstrated that the presence of palmitic and linoleic acids impairs EPC function through a downregulation of AKT/eNOS signal pathway in diabetes.

This mechanism could be one more physiopathological explanation for the overall poor function of EPCs in diabetes [32].

In summary, insulin resistance may adversely affect the contribution of EPCs to endothelial regeneration by several mechanisms such as: 1) reduced NO bioavailability, with subsequent impairment of EPC mobilization from bone marrow, 2) down-regulation of the PI3K/Akt signalling, 3) persistent inflammation which attenuate EPC survival, differentiation and function and 4) free fatty acid liberation [16].

Adiponectin and EPCs

Adiponectin has anti-atherosclerotic and anti-inflammatory effects through its direct actions on vascular cells. The plasma adiponectin levels, which are decreased in type 2 diabetes, correlate inversely with insulin resistance [33].

Adiponectin was found to be capable of increasing both the EPC numbers and migration. Importantly, it has been demonstrated that adiponectin-mediated migration is markedly suppressed by the administration of a PI3K inhibitor, indicating that the effect of adiponectin on the EPC function is mediated in a PI3K-dependent manner [34]. Moreover, adiponectin seems to attenuate EPCs dysfunction and to contribute to the proangiogenic effect of EPCs and EPC-mediated amelioration of vasculopathy.

Advanced Glycation End Products

Advanced glycation end products (AGEs) are a heterogeneous group of products produced by non-enzymatically glycosylation and oxidation after chronic exposure to sugars, associated with diabetes. Once formed, AGEs are, by nature, irreversible. AGEs have been implicated in the pathogenesis of atherosclerotic vasculopathy and several studies suggested that they directly impair the reparative function of EPCs [35]. In fact, recently it has been shown that AGEs inhibit EPCs maturation into sprouting endothelium and increase EPC apoptosis, via increased expression of receptor for AGEs (RAGEs), and activation of p38, extracellular signal-regulated kinases (ERK), mitogen-activated protein kinase pathways and activation of NF-κB. Reduced eNOS expression and suppression of NO release from EPCs appear to be involved in the AGE-induced apoptosis, supporting an important role of eNOS in EPC function and apoptosis in diabetes [36-38].

CONCLUSION

Given the fact that EPCs are impaired, both in number and function, in diabetic patients, an improved understanding of the mechanisms leading to this dysfunction seems to be crucial. It could lead to new strategies to stimulate these progenitor cells and shift the balance to favor maintenance of vascular health in this growing population.

Endothelial dysfunction is one of the earliest events in the cascade of changes that results in target organ damage in patients with diabetes. It has been shown that EPC dysfunction is associated with poor prognosis in diabetic patients. Prolonged exposure to hyperglycemia, insulin resistance and oxidative stress, are some potential mechanisms underlying the altered properties of EPCs in diabetes. Reversal of the underlying processes that cause this impairment of EPCs should be a goal of antidiabetic therapy.

In conclusion, a more extensive understanding of the complex factors regulating EPCs biology in diabetes is warranted in order to develop and investigate more focused clinical therapies for the vascular complications of this growing disease.

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CHAPTER III

Reduced levels of circulating endothelial progenitor cells in acute myocardial infarction patients with diabetes or pre-diabetes: accompanying the glycemic continuum

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Reduced levels of circulating endothelial progenitor cells in acute myocardial infarction patients with diabetes or pre-diabetes: accompanying the glycemic continuum

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Abstract

Background: Diabetic patients have a significantly worse prognosis after an acute myocardial infarction (AMI) than their counterparts. Previous studies have shown that the number of circulating endothelial progenitor cells (EPCs) significantly increase early after an AMI in normoglycemic patients. However, it is well known that type 2 diabetes mellitus (DM) is associated with impaired function and reduced circulating EPCs levels. Nonetheless, few studies have analyzed EPCs response of diabetics to an AMI and the EPC response of pre-diabetic patients has not been reported yet. Therefore, we hypothesized that in the acute phase of an AMI, diabetic and pre-diabetics have lower circulating EPCs levels than patients with normal glucose metabolism. We also evaluated the possible capacity of chronic antidiabetic treatment in the recovery of EPCs response to an AMI in diabetics.

Methods: One-hundred AMI patients were prospectively enrolled in the study. Using the high-performance flow cytometer FACSCanto II, circulating EPCs (CD45dimCD34+KDR+ and CD45dimCD133+KDR+ cells) were quantified, within the first 24 hours of admission. In addition, as an indirect functional parameter, we also analyzed the fraction of EPCs coexpressing the homing marker CXCR4.

Results: We found that in the acute phase of an AMI, diabetic patients presented significantly lower levels of circulating CD45dimCD34+KDR+ and CD45dimCD133+KDR+ EPCs by comparison with nondiabetics, with a parallel decrease in the subpopulations CXCR4+ ($p < 0.001$). Indeed, this study suggests that the impaired response of EPCs to an AMI is an early event in the natural history of DM, being present even in pre-diabetes. Our results, also demonstrated that numbers of all EPCs populations were inversely correlated with HbA1c ($r = -0.432$, $p < 0.001$ for CD45dimCD34+KDR+ cells). Finally, this study suggests that previous chronic insulin therapy (but not oral antidiabetic drugs) attenuate the deficient response of diabetic EPCs to an AMI.

Conclusion: This study indicates that there is a progressive decrease in EPCs levels, from pre-diabetes to DM, in AMI patients. Moreover, glycemic control seems to be determinant for circulating EPCs levels presented in the acute phase of an AMI and chronic insulin therapy may probably attenuate the deficit in EPCs pool seen in diabetics.

Keywords: Endothelial progenitor cells, Diabetes, Pre-diabetes, Insulin, Oral antidiabetic drugs, Acute myocardial infarction, Homing

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Background

It is well recognized that patients with type 2 diabetes mellitus (DM) have accelerated atherosclerosis, increased risk of developing coronary artery disease (CAD) and worse prognosis after an acute myocardial infarction (AMI) [1].

Endothelial progenitor cells (EPCs), a subpopulation of adult stem cells, have emerged as critical to endothelial repair and vascular homeostasis. Although the mechanisms whereby EPCs protect the cardiovascular system are still not fully understood, it has been extensively demonstrated that these bone marrow-derived cells contribute to endothelial repair and postnatal neovascularization [2,3]. EPCs can differentiate into mature endothelial cells and be incorporated into new vessels or act by a paracrine manner, through the secretion of pro-angiogenic growth factors that enhance vascularization mediated by resident endothelial cells and/or promote angiogenesis [2-5].

The number of EPCs in peripheral circulation is generally low, and in normal physiological conditions, these endothelial precursor cells are very rare in blood, but they are mobilized from the bone marrow to the peripheral circulation in response to tissue injury, such as myocardial ischemia [6]. In fact, tissue ischemia is considered the strongest stimulus for EPCs mobilization and it has been shown that their numbers significantly increase in patients with an AMI [7,8]. However, it is well established that diabetic patients present impaired function and reduced numbers of circulating EPCs, reflecting a poor endogenous regenerative capacity that may contribute to the development of vascular complications and to the dismal prognosis associated with this prevalent disease [9-12]. Therefore, it is likely that, in the clinical context of myocardial infarction, diabetic patients also have lower levels of circulating EPCs, but regrettably the data addressing the dynamics of EPCs mobilization in diabetic patients with AMI are scarce. Furthermore, little is known about potential EPCs impairment in pre-diabetic states and no studies are available on the kinetics of EPCs mobilization in pre-diabetic patients with AMI. This is of great importance, since multiple studies have demonstrated that individuals with pre-diabetes are also at increased risk for cardiovascular events [13]. On the other hand, some drugs commonly prescribed in diabetic patients, like statins, angiotensin II receptor blockers (ARBs) and angiotensin-converting-enzyme (ACE)-inhibitors, have been shown to increase the number of EPCs in peripheral blood of patients with stable CAD [14]. However, we have no data available regarding the impact of previous antidiabetic treatment on EPC response to an AMI, in diabetic patients.

In this study, we tested the hypothesis that diabetes and pre-diabetes states were associated with reduced circulating EPCs levels in the acute phase of a myocardial infarction (MI) by comparison with patients with normal glucose metabolism. We also examined the impact of previous antidiabetic treatment on the dynamics of EPCs mobilization in diabetic patients following an AMI.

Methods

Study population and selection

A prospective cohort of 686 consecutive patients hospitalized in a single Coronary Care Unit (CCU) due to myocardial infarction, from 5 January 2009 to 23 September 2011, were screened on admission for inclusion. Screening included an interview, clinical examination, ECG and laboratory assessment. Patients were excluded if they were >80 years old, showed clinical or biochemical evidence of concomitant inflammatory disease, known auto-immune or malignant diseases, severe peripheral arterial occlusive disease, deep vein thrombosis or pulmonary embolism, 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 < 8.5 g/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. A regular use of nonsteroidal anti-inflammatory drugs or anticoagulants, patients with pacemakers, implantable cardioverter defibrillators or resynchronization devices, and excessive alcohol consumption or illicit drugs abuse that may influence EPC kinetics were also exclusion criteria. A total of 100 patients were prospectively included (65% with ST segment elevation myocardial infarction – STEMI and 35% with non-ST segment elevation myocardial infarction - NSTEMI) (Figure 1).

All patients received the standard therapy for the acute phase of MI that included acetylsalicylic acid (ASA), clopidogrel and low-molecular-weight heparin, according to usual hospital practice.

Baseline demographic data, cardiovascular risk factors and previous medications were recorded in all patients. Smoking status was recorded as ever-smoker (past or current) or non-smoker.

Blood samples were collected to assess chemistry (including fasting plasma glucose (FPG) and glycosylated hemoglobin (HbA1C)), total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides, high sensitivity C-reactive protein (hs-CRP), creatinine, and hematological parameters in all patients according to standard hospital practice.

The study was approved by the local ethics committee (Approval Number: HUC-23-08). All patients gave written informed consent and research was conducted according to the principles expressed in the Declaration of Helsinki.

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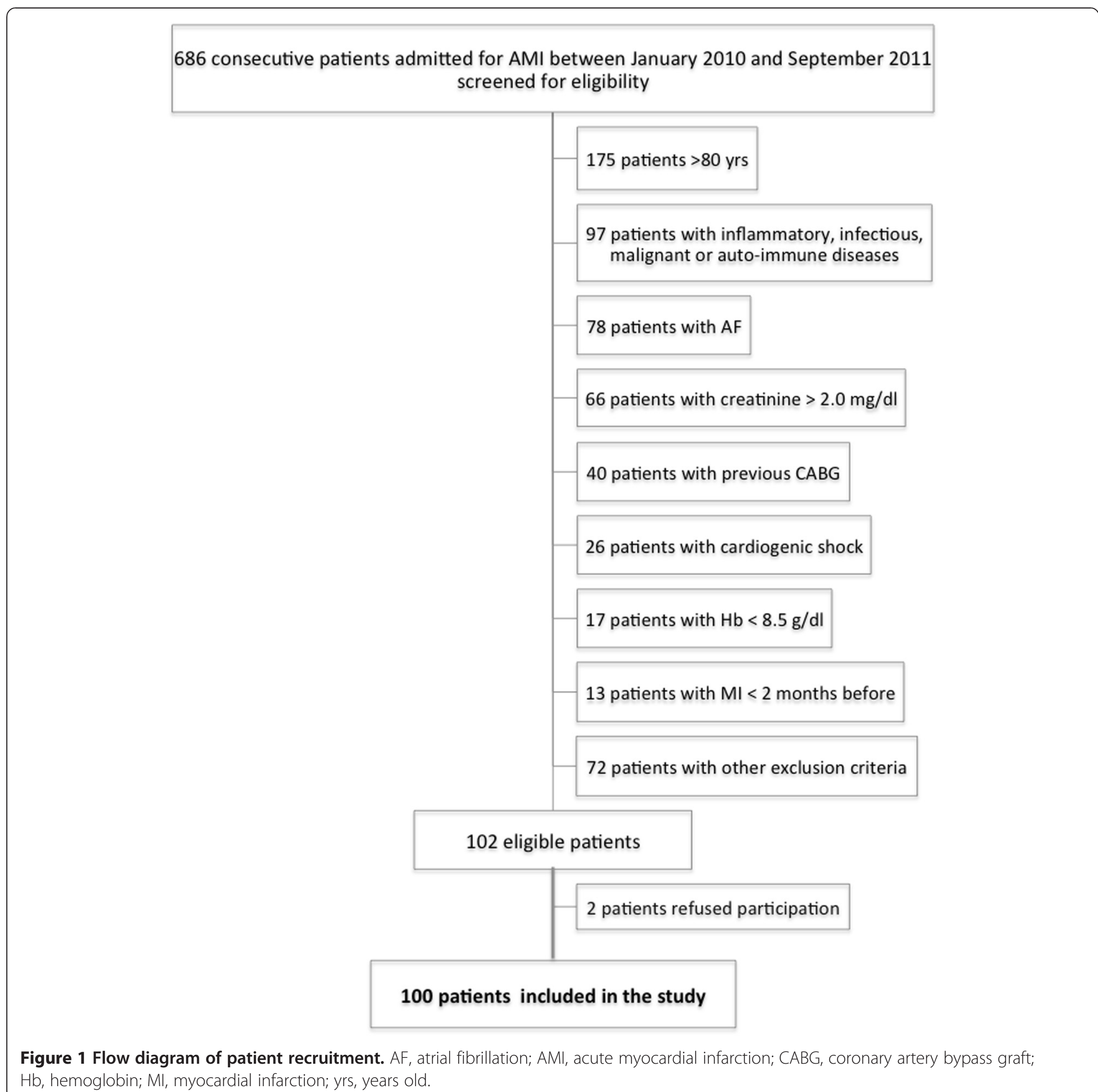

Figure 1 Flow diagram of patient recruitment. AF, atrial fibrillation; AMI, acute myocardial infarction; CABG, coronary artery bypass graft; Hb, hemoglobin; MI, myocardial infarction; yrs, years old.

Classification of glucose metabolism status

DM and glucose metabolism disorders were defined according to the American Diabetes Association (ADA) criteria [15,16]. All patients without previously known diabetes underwent an Oral Glucose Tolerance Test (OGTT) on day 4 or 5 of hospitalization. Therefore, patients were classified as having diabetes if they have a FPG ≥126 mg/dl, a 2-h glucose ≥200 mg/dl on OGTT, a A1c ≥ 6.5% or a random plasma glucose ≥200 mg/dl in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis. For patients without diabetes, prediabetes was defined as FPG levels of 100–125 mg/dl (impaired fasting glucose – IFG), 2-hour OGTT glucose level of 140–199 mg/dl (impaired glucose tolerance - IGT) or HbA1c values of 5.7%-6.4%. Patients were classified as having a normal glucose metabolism (NGM) if they have FPG < 100 mg/dl, 2-hour OGTT glucose level < 140 mg/dl and HbA1c <5.7%.

Quantification of circulating EPCs by flow cytometry

For the identification and quantification of EPCs, we have used a standardized protocol - the modified International Society for Hematotherapy and Graft Engineering (ISHAGE) sequential gating strategy - proposed by

Schmidt-Lucke et al. [17]. Briefly, within the first 24 h of CCU admission, 1 ml of whole blood was collected from a forearm vein into EDTA tubes, transported into the cytometry laboratory and processed within 1 to 2 hours of collection. Hence, 150 µl of whole blood were incubated with the following combination of anti-human monoclonal antibodies: 10 µl of anti-CD133 conjugated with allophycocyanin (APC) (Miltenyi Biotec), 5 µl of anti-CD45 conjugated with APC-H7 (Becton Dickinson), 10 µl of anti-KDR (also known as type 2 vascular endothelial growth factor receptor - VEGF-R2) conjugated with phycoerythrin (PE) (Sigma), 10 µl of anti-CD34 conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson) and 10 µl of anti-CD184 (also known as CXCR4) conjugated with PE-Cyanine 5 (PE-Cy5) (BD Pharmingen) for 30 min at 4°C, in the dark. Red blood cell lysis was performed using FACS Lysing Solution (BDBiosciences) diluted 1:10 (vol/vol) in distilled water and washed with phosphate-buffered-saline (PBS) before flow cytometry acquisition. Data acquisition was performed with a high-performance flow cytometer, a BDBioscience FACSCanto II, which can analyze with high resolution up to eight different fluorescent markers from a large number of events and we used the flow cytometry software Infinicyt 1.5 (Cytognos) for the analysis. According to the used standardized protocol, human circulating EPCs were identified by a minimal antigenic profile that includes at least one marker of stemness/immaturity (CD34 and/or CD133), plus at least one marker of endothelial commitment (KDR). CD45 staining was also performed to exclude leucocytes, as it has been previously demonstrated that only the fraction of CD45dim cells harbours the "true" circulating EPCs [18]. CXCR4, the receptor for stromal cell–derived factor-1 (SDF-1), is a cell surface antigen expressed in EPCs, which plays a key role in their transendothelial migration and homing to sites of vascular injury [19]. Therefore, by analyzing the subpopulation of progenitors coexpressing CXCR4, we could study a functional parameter of EPCs. As isotype controls are known to mask rare cell populations, none were used in this analysis, and baseline fluorescence was determined using unstained cells [20]. Because EPCs are extremely rare events in peripheral blood, additional strategies were applied in order to increase the sensitivity of the method and the accuracy of our work. These included: automatic compensation for minimizing fluorescence spillover, exclusion of dead cells, and use of specific high quality mononuclear antibodies. The total number of acquired events was increased to at least 1 million per sample, which is generally not needed for most other applications of flow cytometry. Circulating EPCs were measured in triplicate from the same patients, revealing a very close correlation ($r = 0.87$, $p < 0.0001$). The same trained operator, who was blind to the clinical status of the patients, performed all the cytometric analysis throughout the study.

Four different populations of EPCs were quantified: 1) CD45dimCD133+KDR+ cells; 2) CD45dimCD34+KDR+ cells; 3) CD45dimCD34+CD133+KDR+ triple positive cells; and 4) the subpopulation of CD45dimCD34+KDR+CXCR4+ EPCs.

Patients follow up for cardiovascular events

All patients were followed up for 24 months after discharge. The following cardiovascular events were recorded: cardiovascular death; nonfatal stroke or transient ischemic attack; re-infarction; unstable angina and re-hospitalization for unstable angina or heart failure. We also analyzed the combined endpoint of cardiovascular death, re-hospitalization for ACS and unplanned PCI – Major Adverse Cardiac Events (MACE). Cardiovascular death was defined as death due to a MI or stroke or documented sudden cardiac death. For patients experiencing more than one acute event, only the first event was considered in the analysis.

Statistical analysis

Statistical analyses were performed using SPSS software version 20.

Based on previous data, we estimated a 40% reduction in circulating EPCs of diabetics by comparison with nondiabetic patients. Therefore, a minimum sample size of 18 patients in each group would provide 90% power to detect difference in circulating EPCs between diabetic and nondiabetic patients, using a two-sided hypothesis test with a significance level (alpha) of 0.05.

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 are 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 Pearson's or Spearman's correlation coefficient, whichever appropriate. Multivariate linear regression analysis was used to assess the relationship between circulating EPCs levels and HbA1c, after adjustment for confounding variables. Kaplan-Meier survival analyses were performed to evaluate time-dependent outcomes. Differences between pairs of survival curves were tested by the log-rank test. For all analyses, a 2-sided value of $P < 0.05$ was considered statistically significant.

Table 1 Comparison of clinical characteristics between diabetic and nondiabetic patients

	Non-diabetics (N = 62)	Type 2 diabetics (N = 38)	p value
Age (years)*	59.8 ± 10.3	61.5 ± 11.0	0.300
Male gender (%)	90.3	89.5	0.891
BMI (Kg/m^2)*	27.9 ± 4.4	29.2 ± 6.9	0.251
Previous CAD (%)	14.5	31.6	0.075
Previous MI (%)	11.3	18.4	0.319
Type of MI			
STEMI vs NSTEMI (%)	66.1/33.9	63.2/36.8	0.762
Cardiovascular risk factors			
Hypertension (%)	56.5	84.2	0.004
Smoking habits (%)	61.3	47.4	0.215
Family history (%)	37.1	28.9	0.492
Hyperlipidemia (%)	71.1	82.3	0.189
Physical inactivity (%)	56.5	60.5	0.689
Previous cardiovascular or antidiabetic drugs			
Statins (%)	29.0	31.6	0.825
ASA (%)	19.4	42.1	0.021
ACEI (%)	12.9	36.8	0.007
ARB (%)	12.9	31.6	0.038
Beta-blockers (%)	9.7	21.1	0.141
Insulin (%)	0.0	26.3	<0.001
Oral hypoglycemic (%)	0.0	65.8	<0.001
Baseline laboratory			
Admission Troponin I (μg/L)§	0.7 ± 5.8	1.5 ± 3.6	0.798
Peak Troponin I (μg/L)§	55.4 ± 71.6	56.7 ± 64.7	0.793
HbA1C (%)§	5.6 ± 0.5	7.1 ± 2.2	<0.001
Admission glycemia (mg/dl)§	109.0 ± 31.0	206.5 ± 110.8	<0.001
First fasting glycemia (mg/dl)§	103.0 ± 24.5	156.0 ± 52.5	<0.001
Total cholesterol (mg/dl)*	178.5 ± 59.0	211.7 ± 54.9	0.007
LDL cholesterol (mg/dl)*	113.2 ± 39.6	145.3 ± 44.3	<0.001
HDL cholesterol (mg/dl)§	40.2 ± 9.5	38.7 ± 12.9	0.164
Triglycerides (mg/dl)§	138.5 ± 109.5	148.0 ± 88.5	0.801
Uric acid (mg/dl)*	5.6 ± 1.3	6.2 ± 1.4	0.096
Baseline creatinine (mg/dl)§	0.8 ± 0.3	0.9 ± 0.4	0.123
Baseline hemoglobin (g/dl)*	14.8 ± 1.4	14.4 ± 1.2	0.200
Admission hs-CRP (mg/dl)*	0.9 ± 1.3	1.0 ± 1.4	0.872
LVEF (%)*	52.6 ± 9.6	50.0 ± 11.8	0.104
Hospital length of stay§	5.4 ± 2.6	5.9 ± 3.0	0.424

ACEI, Angiotensin-Converting Enzyme Inhibitors; ARB, Angiotensin II receptor blockers; ASA, acetylsalicylic acid; CAD, coronary artery disease; hs-CRP, high sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; MI, myocardial infarction; STEMI, ST elevation myocardial infarction; NSTEMI, non-ST elevation myocardial infarction.
*mean ± SD.
§median ± interquartile range.

Results

Characteristics of the study population

There were 38 patients with DM, 13% of them with newly diagnosed DM. Overall, diabetics had similar age and cardiovascular risk factors as nondiabetic patients, except for hypertension that was significantly more frequent in diabetics (Table 1). Additionally, they tended to have more frequently previously known CAD and were more often

medicated with ASA, ACE-inhibitors and ARB as well as oral hypoglycemic agents and insulin before admission than nondiabetics. As expected, diabetics had significantly higher levels of admission glycemia, fasting glycemia and HbA1c and also presented higher total cholesterol and LDL-cholesterol than nondiabetics.

There were no significant differences in MI presentation (STEMI versus NSTEMI), left ventricular function or renal function between groups (Table 1).

There were no significant differences in the extent of coronary atherosclerosis, number of stents deployed or other cath lab parameters between diabetics and nondiabetics (Table 2).

Reduction of circulating EPCs in diabetic patients

Circulating EPCs levels were expressed for one million cytometric events (Figure 2). Diabetic patients had circulating numbers of $CD45^{dim}$CD34+KDR+ cells reduced by 63% when compared with nondiabetics, with a parallel decrease in the subpopulation CXCR4+ (Table 3, Figure 3). There was also a significant reduction in the more immature population of CD45dimCD34+CD 133+KDR+ EPCs to around half the levels of nondiabetics, and numbers of its precursors CD45dimCD13 3+KDR+ in peripheral circulation were also significantly decreased. The subpopulation coexpressing the homing marker CXCR4 (CD45dimCD133+KDR+CXCR4+) was also significantly reduced in diabetics (Table 3).

Circulating EPCs levels across the different disorders of glucose metabolism

Upon OGTT, 24 of the nondiabetic patients had pre-diabetes (29.2% with impaired fasting glucose - IFG, 58.3% with impaired glucose tolerance – IGT and 12.5% with both disorders of glucose metabolism).

Circulating CD45dimCD34+KDR+ EPCs decreased as a continuum from NGM to DM, as there was a reduction of approximately 40% in patients with pre-diabetes as compared with NGM patients (p = 0.018) and there was an additional reduction of these EPCs of about 40% (p = 0.042) when diabetics were compared with patients with pre-diabetes (Table 4). Nonetheless, the population of more immature progenitor cells (CD45dimCD133 +KDR+) and the subpopulations coexpressing the CXCR4 marker (CD45dimCD34+KDR+CXCR4+ and CD45dimCD 133+KDR+CXCR4+) were not significantly reduced in pre-diabetic patients by comparison with NGM patients (5.4 ± 2.4 vs 3.9 ± 2.8, p = 0.314; 1.8 ± 0.9 vs 1.3 ± 1.2, p = 0.175; and 3.5 ± 2.1 vs 3.2 ± 1.3, p = 0.290, respectively), whereas a significant reduction was apparent from pre-diabetic to diabetic patients on these cells levels (p = 0.022; p = 0.045 and p = 0.015, respectively) (Table 4).

Circulating EPCs numbers according to previous antidiabetic treatment

Regarding the antidiabetic strategy before admission, there were 53% of diabetic patients on oral hypoglycemic drugs, 26% insulin-treated diabetics, and 21% of patients who were not taking any antidiabetic drug (because they were on diet-only therapy or new onset DM was diagnosed during hospitalization). As expected, diabetes duration was significantly longer in insulin-treated patients (13.5 ± 9.8 years versus 6.8 ± 5.0 in patients on oral hypoglycemic drugs versus 1.7 ± 1.2 in diabetics not receiving any antidiabetic drug, p = 0.001). Insulin-treated DM (ITDM) patients and diabetics not previously treated with antidiabetic drugs presented a worse glycemic control as compared with patients on oral hypoglycemic drugs (Figure 4).

Numbers of CD45dimCD34+KDR+ EPCs were significantly reduced in diabetic patients previously treated with oral antidiabetic drugs and in diabetics not taking any hypoglycemic drug when compared with nondiabetic patients (Figure 5, A). However, despite the worse

Table 2 Comparison of catheterization lab data between diabetics and nondiabetics

	Non-diabetics (N = 62)	Type 2 diabetics (N = 38)	p value
Catheterization during hospitalization (%)	94.7	96.8	0.614
Normal coronaries (%)	5.0	0.0	0.289
1-vessel disease (%)	41.7	41.7	1.000
2-vessel disease (%)	26.7	30.6	0.682
3-vessel disease (%)	26.7	27.8	0.906
Left main disease (%)	6.7	5.7	0.854
LAD disease (%)	69.5	80.6	0.235
PCI before EPCs evaluation (%)	72.6	68.4	0.656
Complete revascularization before EPCs evaluation (%)	44.9	45.3	0.912
Number of stents deployed before EPCs evaluation	1.6 ± 1.1	1.8 ± 1.2	0.649

LAD; left anterior descending, PCI; percutaneous coronary intervention.

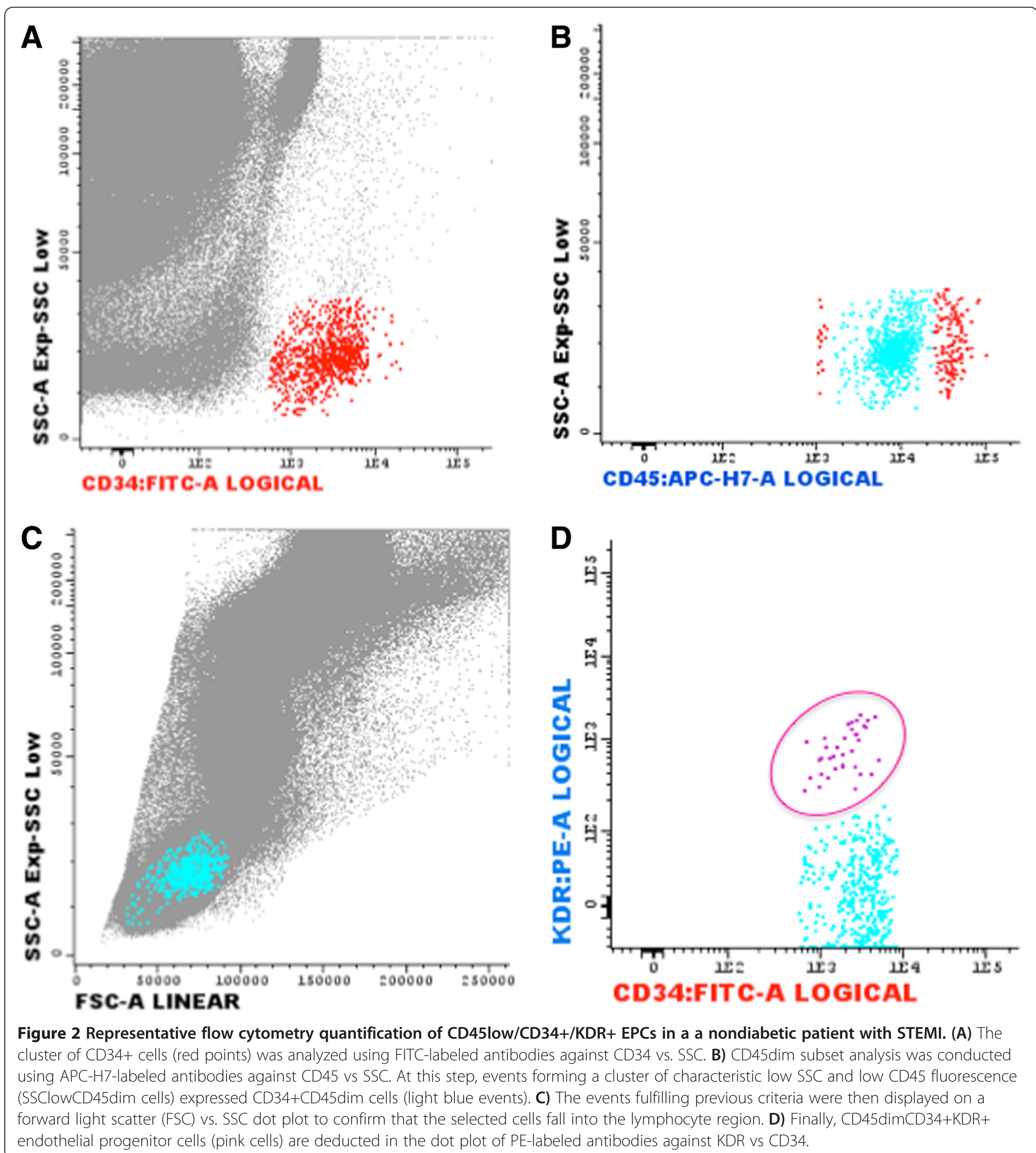

Figure 2 Representative flow cytometry quantification of CD45low/CD34+/KDR+ EPCs in a a nondiabetic patient with STEMI. (A) The cluster of CD34+ cells (red points) was analyzed using FITC-labeled antibodies against CD34 vs. SSC. **B)** CD45dim subset analysis was conducted using APC-H7-labeled antibodies against CD45 vs SSC. At this step, events forming a cluster of characteristic low SSC and low CD45 fluorescence (SSClowCD45dim cells) expressed CD34+CD45dim cells (light blue events). **C)** The events fulfilling previous criteria were then displayed on a forward light scatter (FSC) vs. SSC dot plot to confirm that the selected cells fall into the lymphocyte region. **D)** Finally, CD45dimCD34+KDR+ endothelial progenitor cells (pink cells) are deducted in the dot plot of PE-labeled antibodies against KDR vs CD34.

glycemic control of diabetics on chronic insulin, their CD45dimCD34+KDR+ EPCs levels were not significantly reduced compared to that of nondiabetic patients ($p = 0.160$) (Figure 5-A). Regarding the subpopulation of CD45dimCD34+KDR+ cells also expressing the homing marker CXCR4+, all diabetes treatment categories presented significantly decreased circulating levels by comparison with nondiabetic patients (Figure 5-B). Circulating CD45dimCD133+KDR+ cell levels showed a progressive decline from nondiabetics, untreated DM, DM on oral hypoglycemic drugs and finally, ITDM, with patients receiving insulin and patients on oral hypoglycemic drugs presenting significantly lower levels as compared with nondiabetics ($p = 0.002$ and $p = 0.004$,

Table 3 Comparison of circulating EPCs levels between diabetics and nondiabetics

	Non-diabetics (n = 62)	Type 2 diabetics (n = 38)	p value
Time from PCI to blood sampling (hours)	13.8 ± 14.7	11.6 ± 11.7	0.649
CD34+ cells/10^6 WBC	228.8 ± 136.7	197.0 ± 115.2	0.098
CD133+/10^6 WBC	54.4 ± 35.7	36.0 ± 18.0	0.020
CD45dimCD34+KDR+ cells/10^6 WBC	6.2 ± 3.0	2.3 ± 0.9	<0.001
CD45dimCD34+KDR+CXCR4+ cells/10^6 WBC	1.8 ± 1.1	0.8 ± 0.7	<0.001
CD45dimCD34+CD133+KDR+ cells/10^6 WBC	2.1 ± 1.1	1.0 ± 0.8	<0.001
CD133+KDR+/10^6 WBC	4.6 ± 2.9	3.1 ± 1.6	<0.001
CD133+KDR+CXCR4+/106 WBC	3.5 ± 1.9	2.0 ± 1.2	<0.001

PCI, percutaneous coronary intervention; WBC, white blood cells.

respectively) (Figure 5-C). Circulating levels of the CD4 5dimCD133+KDR+CXCR4+ subpopulation were also significantly lower in all diabetic treatment categories than in nondiabetic patients (Figure 5-D).

Impact of glycemic control on EPCs levels

There were significant negative correlations between levels of circulating CD45dimCD34+KDR+ (Figure 6, A), CD45dimCD133+KDR+ progenitors (Figure 6, C), their CXCR4+ subpopulations (Figure 6, B and D) and HbA1c. CD45dimCD34+KDR+EPCs and their subpopulation CD45 dimCD34+KDR+CXCR4+ were also inversely correlated with fasting glycemia ($r = -0.371$, $p < 0.001$ and $r = -0.213$, $p = 0.046$, respectively). Nonetheless, EPCs levels were not correlated with DM duration. Levels of circulating CD45dimCD34+KDR+ and CD45dimCD133+KDR+ progenitors were also negatively correlated with age ($r = -0.285$, $p = 0.007$ and $r = -0.343$, $p = 0.001$, respectively).

Remarkably, correlations with HbA1c remain significant even after adjustment for age, gender, hypertension, LDL-cholesterol, family history of CAD, smoking habits and physical inactivity (Table 5).

Prognostic impact of EPCs

Clinical outcomes during the 24 months follow-up period are represented in Table 6.

There were no significant differences in re-infarction, nonfatal stroke/transient ischemic attack or cardiovascular

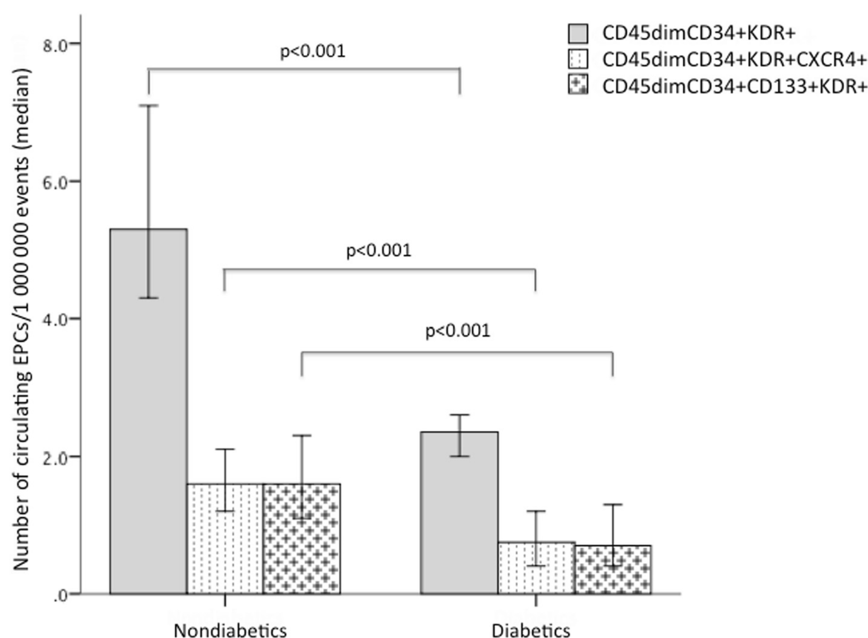

Figure 3 Comparison of circulating EPCs levels between diabetics and nondiabetics. Bars represent median and error bars interquartile range of circulating EPCs numbers quantified by flow cytometry. Mann Whitney U test was used for the comparison between diabetic and nondiabetic EPCs levels.

Table 4 Comparison of circulating EPCs levels between the different glucose metabolism status

	NGM (n = 38)	Pre-diabetes (n = 24)	Diabetes (n = 38)	p value
CD34+ cells/10^6 WBC	417.3 ± 266.9	225.4 ± 97.5	176.5 ± 148.8	0.006
CD133+/10^6 WBC	41.5 ± 23.7	34.1 ± 21.2	34.4 ± 19.2	0.101
CD45dimCD34+KDR+ cells/10^6 WBC	7.0 ± 3.5	4.3 ± 2.7	2.4 ± 1.2	<0.001
CD45dimCD34+KDR+CXCR4+ cells/10^6 WBC	1.8 ± 0.9	1.3 ± 1.2	0.8 ± 0.7	0.002
CD45dimCD34+CD133+KDR+ cells/10^6 WBC	1.7 ± 1.0	1.3 ± 1.1	0.7 ± 0.6	0.001
CD133+KDR+/10^6 WBC	5.4 ± 2.4	3.9 ± 2.8	3.0 ± 1.9	0.002
CD133+KDR+CXCR4+/10^6 WBC	3.5 ± 2.1	3.2 ± 1.3	2.0 ± 1.4	0.002

NGM, normal glucose metabolism; WBC, white blood cells.

mortality rates between groups. However, the occurrence of unstable angina, the composite endpoints MACE and re-hospitalization for unstable angina or heart failure were significantly higher in diabetics, with the following odds ratios 6.89 (95% CI, 1.35-35.19), 4.23 (95% CI 1.43-12.53) and 4.82 (95% CI 1.52-15.30), respectively.

Regarding baseline circulating EPCs levels, patients with unstable angina, unplanned PCI or MACE during follow-up presented significantly lower levels of CD45dimCD34+KDR+ and CD45dimCD133+KDR+ EPCs. Levels of the CD45dimCD133+KDR+CXCR4+ EPCs subpopulation were also significantly reduced, at baseline, in patients who underwent unstable angina or MACE during the 2-year follow-up period (Figure 7). Additionally, the Kaplan–Meier survival curves for freedom from MACE according to EPCs levels showed a significantly lower event-free survival rate in patients with lower EPCs levels in the early phases of AMI (log-rank test, p = 0.023 for CD45dimCD34+KDR+ EPCs and log-rank test, p = 0.004 for CD45dimCD133+KDR+ cells) (Figure 8).

Discussion

There were four major findings in the present study. First, we confirmed that, in the acute phase of a MI, diabetic patients present dramatically reduced levels of circulating EPCs by comparison with nondiabetics. Second, this study showed for the first time that even pre-diabetes reduces EPCs response to an AMI, since EPCs levels were significantly reduced in pre-diabetics and further reduced in diabetics as compared with patients with NGM. Third,

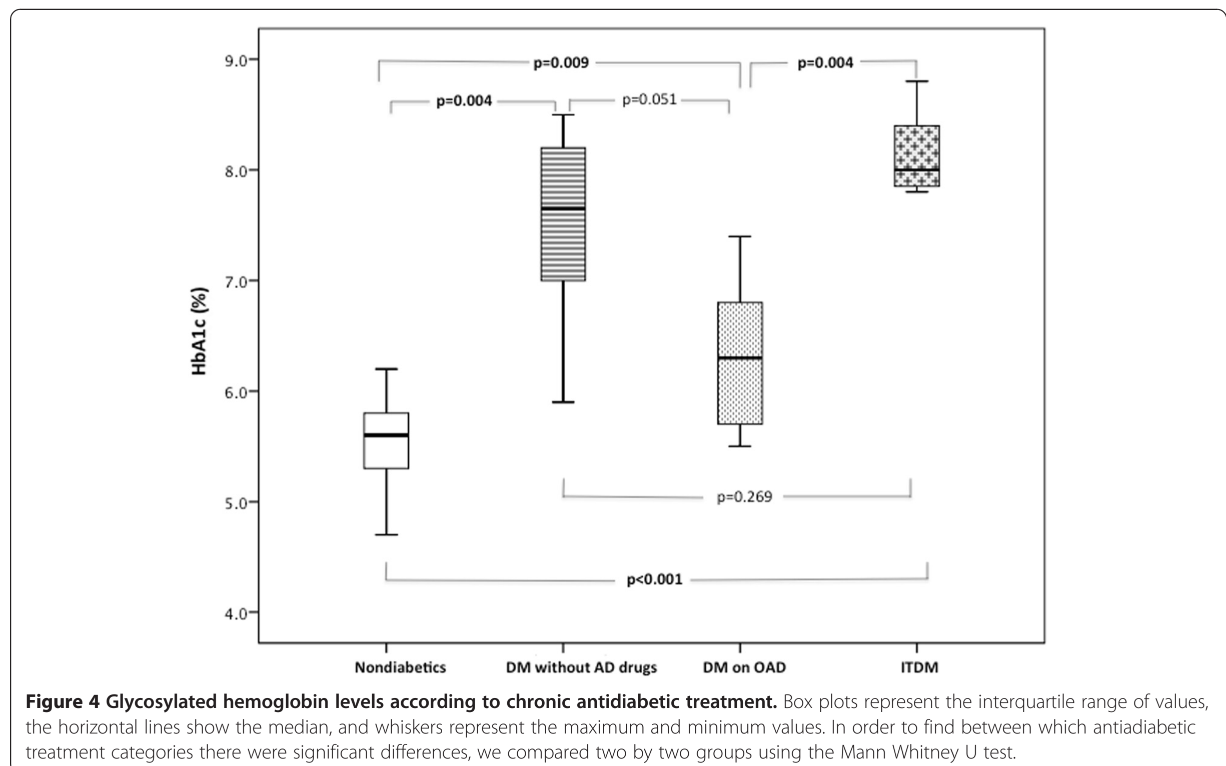

Figure 4 Glycosylated hemoglobin levels according to chronic antidiabetic treatment. Box plots represent the interquartile range of values, the horizontal lines show the median, and whiskers represent the maximum and minimum values. In order to find between which antiadiabetic treatment categories there were significant differences, we compared two by two groups using the Mann Whitney U test.

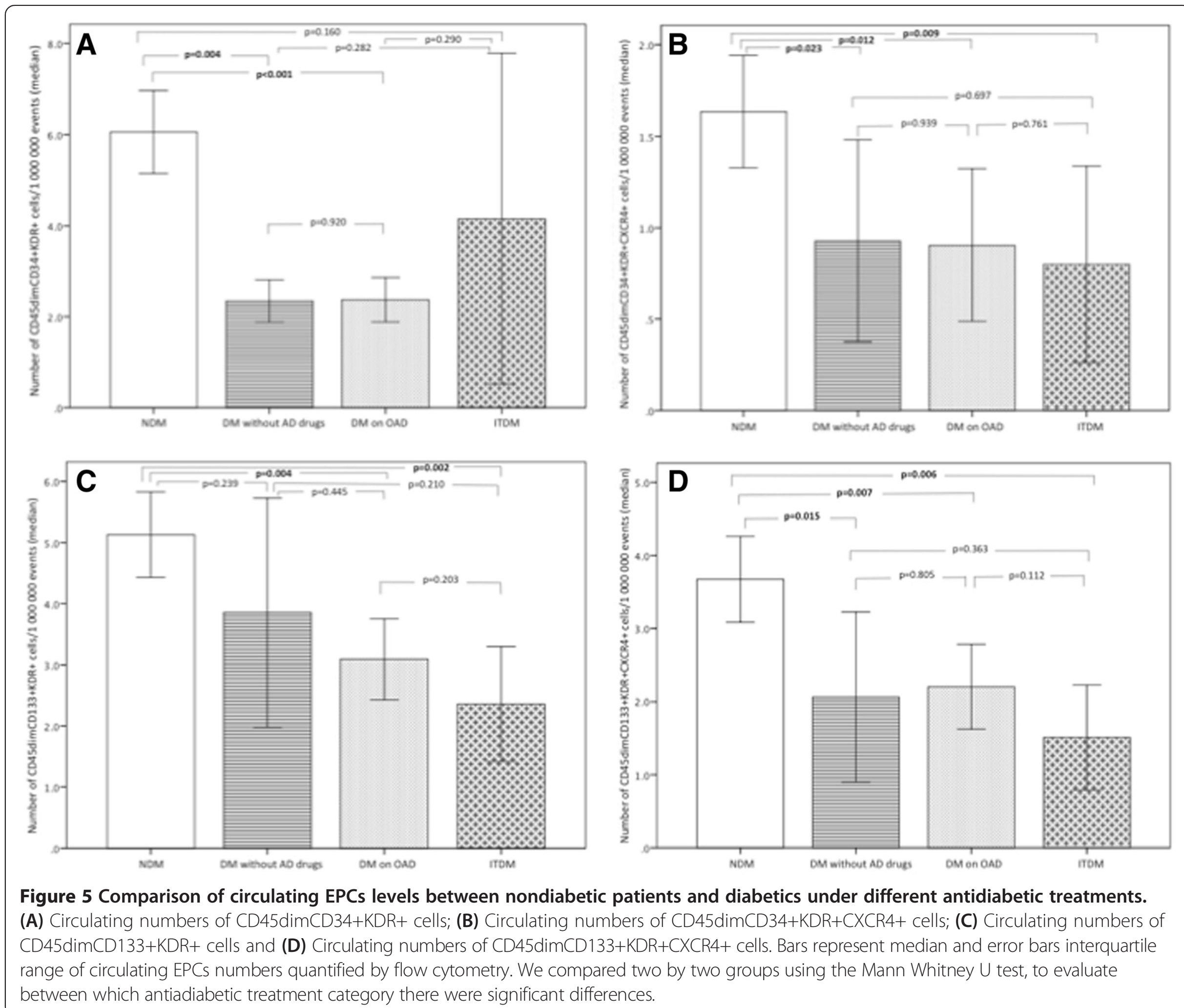

Figure 5 Comparison of circulating EPCs levels between nondiabetic patients and diabetics under different antidiabetic treatments. **(A)** Circulating numbers of CD45dimCD34+KDR+ cells; **(B)** Circulating numbers of CD45dimCD34+KDR+CXCR4+ cells; **(C)** Circulating numbers of CD45dimCD133+KDR+ cells and **(D)** Circulating numbers of CD45dimCD133+KDR+CXCR4+ cells. Bars represent median and error bars interquartile range of circulating EPCs numbers quantified by flow cytometry. We compared two by two groups using the Mann Whitney U test, to evaluate between which antiadiabetic treatment category there were significant differences.

previous chronic insulin therapy (but not oral antidiabetic drugs) seems to attenuate the deficit in circulating EPCs seen in diabetic patients with an AMI. Finally, we have demonstrated that the degree of glycemic control is an important determinant of circulating EPCs numbers in the setting of an AMI.

An AMI is a recognized pathological stimulus for EPCs mobilization. In fact, patients with AMI present significantly increased numbers of circulating EPCs as compared with control subjects or with patients with stable angina [8]. It has been shown that circulating EPCs increase immediately after the onset of an AMI, with a subsequent peak at day 5 and a rapid decline thereafter, normalizing within 2 months [21,22]. Circulating EPCs constitute a key endogenous repair mechanism to counteract ongoing endothelial cell injury, replace dysfunctional endothelium, and enhance tissue repair after ischemic vascular injury [23]. Of note, depletion of circulating EPCs pool and impaired migratory activity of these progenitor cells have been shown to be predictive of future adverse cardiovascular events [24,25]. In accordance with these previous studies, our work showed that freedom from MACE following an AMI was significantly poorer in patients with lower baseline EPCs levels.

It has been extensively demonstrated that patients with DM have a profound reduction of EPCs levels in peripheral blood, which has been correlated with the high cardiovascular morbidity and mortality associated with diabetes [10,26]. Additionally, reduced EPCs numbers have been independently associated with impaired myocardial function in diabetic patients [27]. Fadini et al. have demonstrated in diabetic animals, a deficient EPCs mobilization and impaired compensatory angiogenesis after hindlimb ischaemia-reperfusion injury [28]. However, in the clinical setting of AMI, and despite the important vascular protective role of EPCs, to date, only

António *et al. Cardiovascular Diabetology* 2014, **13**:101
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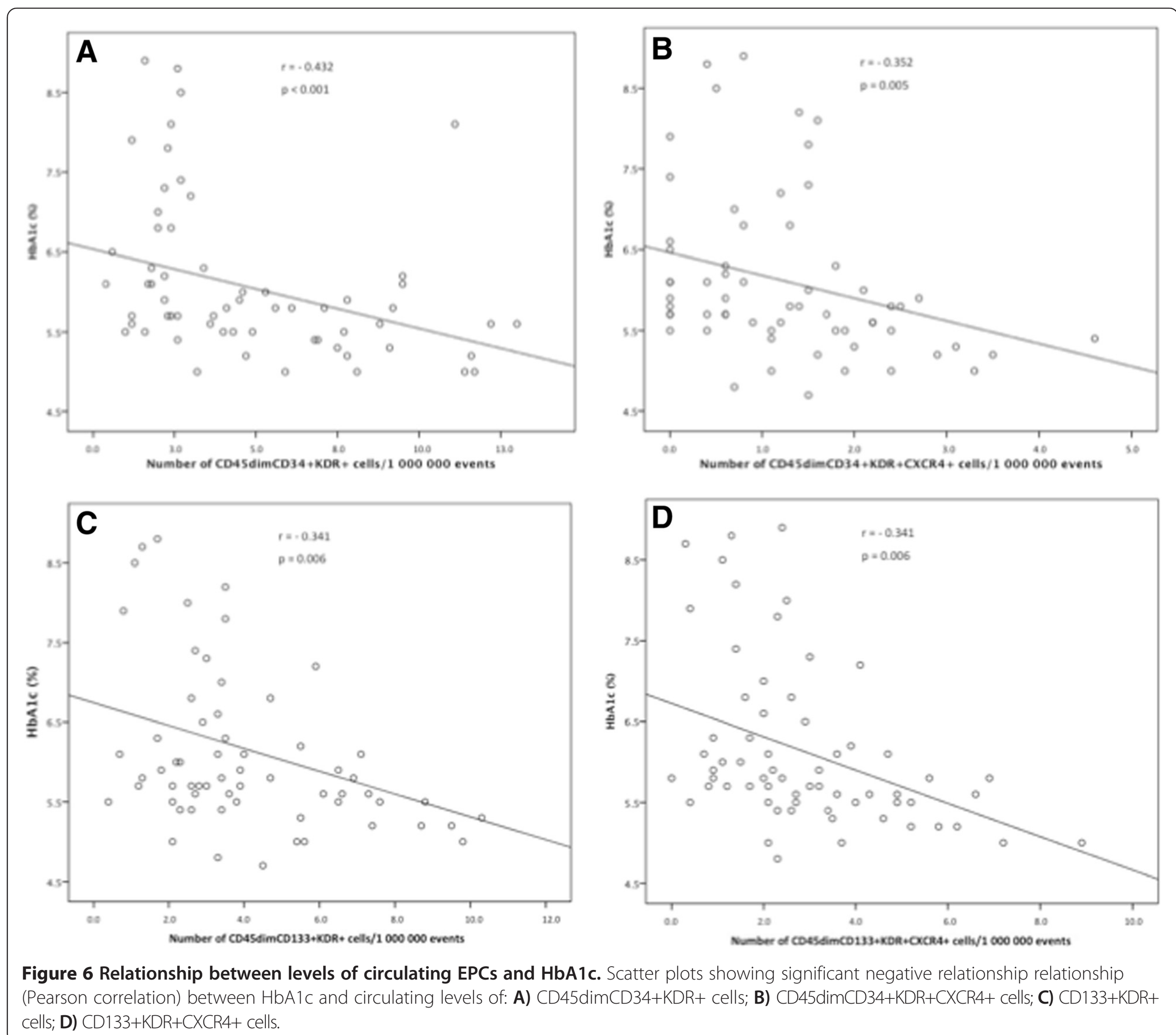

Figure 6 Relationship between levels of circulating EPCs and HbA1c. Scatter plots showing significant negative relationship relationship (Pearson correlation) between HbA1c and circulating levels of: **A)** CD45dimCD34+KDR+ cells; **B)** CD45dimCD34+KDR+CXCR4+ cells; **C)** CD133+KDR+ cells; **D)** CD133+KDR+CXCR4+ cells.

three clinical studies have studied the dynamics of EPCs mobilization in diabetic patients [21,22,29]. In those studies, circulating EPCs levels were decreased in diabetics [21,22] (or hyperglycemic patients, in the Marfella et al. study) [29] compared with non-diabetic patients immediately after the onset of AMI (day 1). Moreover, it has been demonstrated that the peak level of circulating EPCs was delayed in diabetic patients compared with that of nondiabetic patients (from day 5 in nondiabetic patients to day 7 in diabetic patients) [21,22]. Consistent with these previous studies, the present work confirmed that circulating EPCs levels were strikingly reduced in the early phases of an AMI in diabetic patients as compared with nondiabetic patients. Of note, this importantly reduction in EPCs levels seen in diabetic patients does not seem justified by differences in myocardial ischemia or different coronary revascularization procedures between groups, as values of troponin I (a highly specific marker of myocardial injury) and coronary revascularization were similar in diabetic and nondiabetic patients.

It has become evident that circulating EPCs numbers were inversely correlated to the severity of CAD [30,31]. However, in the present study the huge difference in EPCs levels between AMI diabetics and nondiabetics cannot be explained by differences in CAD severity, since there were no significant differences in the extension of coronary stenosis between both groups.

A large body of evidence links classical cardiovascular risk factors, such as hypertension, with reduction in circulating EPCs [32]. In this study population, diabetics presented a significantly higher prevalence of hypertension that could exacerbate the difference in EPCs levels as compared with nondiabetics. However, diabetics were also more frequently treated with drugs that recognizably

Table 5 Multivariate regression analysis assessing the correlation between HbA1c and circulating progenitor cells levels, after adjustment for other cardiovascular risk factors than diabetes

Variable	CD45dimCD34+KDR+ levels		CD45dimCD34+KDR+CXCR4+ levels		CD133+KDR+ levels		CD133+KDR+CXCR4+ levels	
	Standard coefficient (ß)	p	Standard coefficient (ß)	p	Standard coefficient (ß)	p	Standard coefficient (ß)	p
HbA1c	−0.308	0.019	−0.260	0.031	−0.342	0.009	−0.416	0.001
Age	−0.188	0.217	−0.107	0.482	−0.254	0.090	−0.052	0.720
Gender	0.208	0.067	0.207	0.119	0.044	0.740	0.158	0.233
Hypertension	−0.071	0.597	−0.075	0.579	0.025	0.855	−0.015	0.905
LDL-cholesterol	−0.057	0.683	−0.047	0.727	−0.176	0.202	−0.148	0.267
Family history of CAD	−0.129	0.361	−0.170	0.202	0.081	0.560	0.078	0.563
Smoking habits	0.003	0.985	−0.204	0.188	−0.086	0.563	−0.133	0.369
Physical inactivity	−0.203	0.139	−0.080	0.556	−0.057	0.657	−0.167	0.180
Adjusted R^2	0.264	…	0.256	…	0.246	…	0.247	…
Significance (ANOVA)	…	0.032	…	0.040	…	0.046	…	0.033

CAD, coronary artery disease; HbA1c, hemoglobin A1c; LDL, low density lipoprotein.

increase circulating EPCs numbers, such as ACE-inhibitors and ARBs, what would counterbalance the possible reduction on EPCs numbers due to the higher prevalence of hypertension [14].

EPCs can be identified on the basis of the expression of surface markers, by flow cytometry, a method considered the gold standard for the quantification of these cells in peripheral blood [33]. Of note, there are no unique or specific surface antigen that can be used to identify circulating EPCs. Therefore, FACS protocols must use the combination of various membrane markers for EPCs quantification. In the present work, we used a standardized polychromatic FACS protocol based upon the detection of CD34 (an adhesion molecule expressed mainly on haematopoietic stem cells) [34], CD133/AC 133 (a surface marker expressed in an immature subset of EPCs, which share more characteristics of stem/progenitor cells) [35], KDR/VEGF-R2 (a typical endothelial marker) [36], CXCR4/CD184 (a homing marker) [19] and CD45dim (critical to exclude myeloid cells and because it has been previously demonstrated that only the fraction of CD45dim cells harbors the "true" circulating EPCs) [17,18]. Importantly, there are no studies in the literature that have attempted to quantify, at the same time, both CD45dimCD34+KDR+EPCs and the more immature population of CD45dimCD133+KDR+ progenitors in patients with AMI. Thus, until now there has been no data available on the relation between these 2 populations in diabetics with an AMI, which would be important to elucidate the mechanisms underlying their impaired response. In this study, we showed for the first time that, not only CD45dimCD34+KDR+ but also 2 the more immature precursors CD45dimCD34+CD133+KDR+ and CD45dimCD133+KDR+ were significantly reduced in diabetic AMI patients by comparison with nondiabetics. Based on these results, it is tempting to speculate that EPCs reduction in diabetes was due, at least in part, to impaired bone marrow mobilization. Because, if the reduction in EPCs levels was motivated by a decrease in survival alone it would be expected to have reduced levels of CD45dimCD34+KDR+ but increased, or at least normal, levels of the more immature population of CD45dimCD133+KDR+ cells, due to positive feedback stimulation of bone marrow recruitment. What we verified here was that the reduction in the more mature EPCs population was not accompanied by the expected up regulation of the more immature ones. In fact, despite the reduction in CD45dimCD34+KDR+

Table 6 Comparison of clinical outcomes after AMI between diabetics and nondiabetics

	Nondiabetics (N = 62)	Diabetics (N = 38)	Odds ratio	P value
Cardiovascular mortality (%)	1.6	7.9	5.2	0.120
Stroke or TIA (%)	0	5.4	-	0.064
Re-infarction (%)	3.3	0	-	0.266
Unstable Angina (%)	3.3	18.9	6.9	0.009
Re-hospitalization for UA or HF (%)	8.1	29.7	4.8	0.005
MACE (%)	9.8	31.6	4.2	0.006

AMI, acute myocardial infarction; HF, heart failure; MACE, Major Adverse Cardiac Events; TIA, transient ischemic accident; UA, unstable angina.

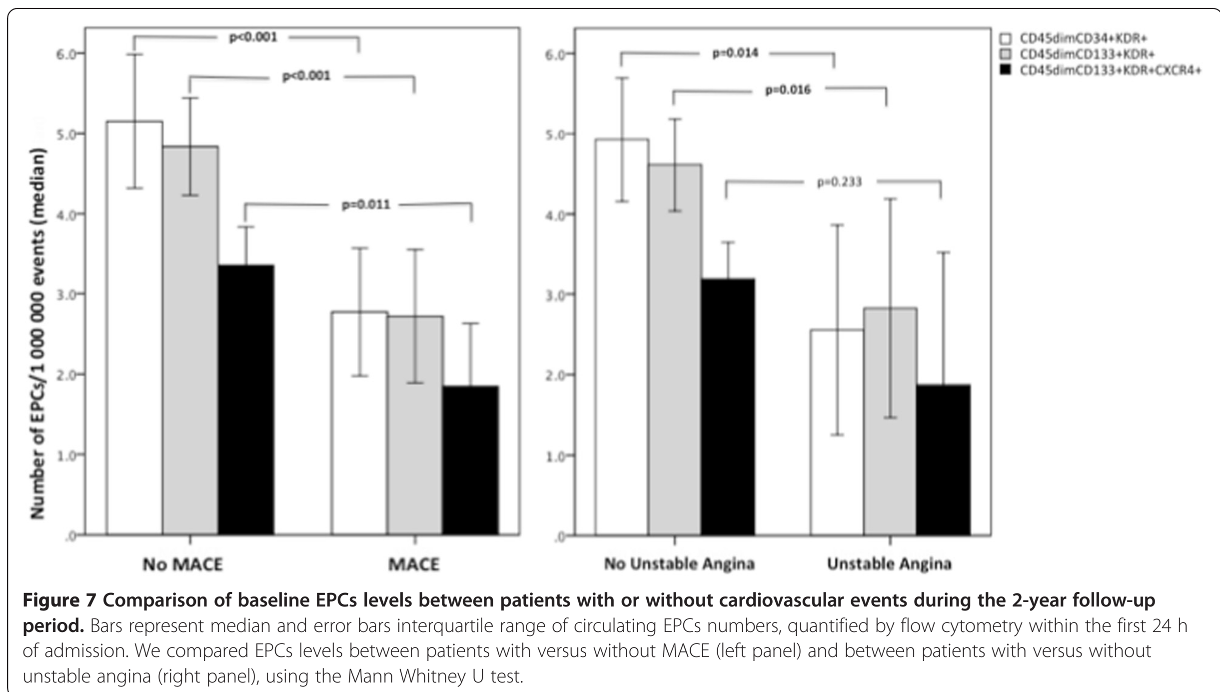

Figure 7 Comparison of baseline EPCs levels between patients with or without cardiovascular events during the 2-year follow-up period. Bars represent median and error bars interquartile range of circulating EPCs numbers, quantified by flow cytometry within the first 24 h of admission. We compared EPCs levels between patients with versus without MACE (left panel) and between patients with versus without unstable angina (right panel), using the Mann Whitney U test.

levels, CD45dimCD133+KDR+ and CD45dimCD34+CD133+KDR+ precursors were also reduced, pointing to impairment in recruitment mechanisms.

Besides the reduction in EPCs counts, we found that the fraction of EPCs coexpressing the homing receptor CXCR4 were also significantly reduced in diabetic AMI patients what may represent an impaired homing capacity of these cells to sites of vascular damage. In fact, CXCR4, the only known receptor for SDF-1, has been reported to play an important role in EPCs homing [19]. Moreover, CXCR4/SDF-1 interaction influences proliferation and mobilization of EPCs from the bone marrow [37]. Since functional study of EPCs, in large populations, with in vitro assays is prohibitively expensive and time consuming, the analysis by flow cytometry of EPCs coexpressing CXCR4 may provide a promising alternative parameter to assess EPCs function. This is the first study to show a reduction in numbers of EPCs coexpressing CXCR4 in diabetic patients with AMI compared with AMI nondiabetics. It is probable that this down regulation in CXCR4+ cells denotes a homing impairment, which in addition to the markedly reduction in circulating EPCs levels may contribute to the worsened outcome post-AMI observed in diabetics.

Pre-diabetes is a general term that refers to an intermediate stage between NGM and overt DM, including IFG and IGT. These disorders of glucose metabolism confer an increased risk for developing both DM and cardiovascular events [13,15,38]. In the present study we have found that CD45dimCD34+KDR+ EPCs were significantly lower in pre-diabetic patients and further reduced in those with DM, as compared with individuals with NGM, suggesting that the reduction in the more mature EPCs population follows the continuum of DM development. These findings suggest that circulating EPCs reduction is an early event in the natural history of DM, what is in accordance to a previous work of Fadini et al. [39]. That study has shown, in individuals from a metabolic outpatient clinic, that circulating CD34+KDR+ cells present a progressive decline from NGM, to prediabetics and diabetic patients and that both fasting and post-challenge glucose were inversely related to circulating CD34+KDR+ EPCs levels [39]. Our work further extends these findings by the quantification of more immature EPCs populations and the study of homing function by the analysis of CXCR4+ subpopulations. Interestingly, we verified that CD45dimCD133+KDR+ EPCs and both subpopulations of CXCR4+ EPCs (CD45dimCD34+KDR+CXCR4+ and CD45dimCD133+KDR+CXCR4+ cells) were not significantly reduced in pre-diabetic AMI patients, compared to patients with NGM. One possible explanation for this divergent influence on different EPCs populations is that pre-diabetes reduces EPCs survival (with subsequent reduction CD45dimCD34+KDR+ EPCs levels) but, does not impair neither bone marrow recruitment of EPCs (leading to no differences in levels of CD45dimCD133+KDR+ EPCs) nor homing processes (explaining the normal proportion of EPCs coexpressing CXCR4).

Previous *in vitro* and several animal studies have demonstrated that insulin therapy has a protective role over

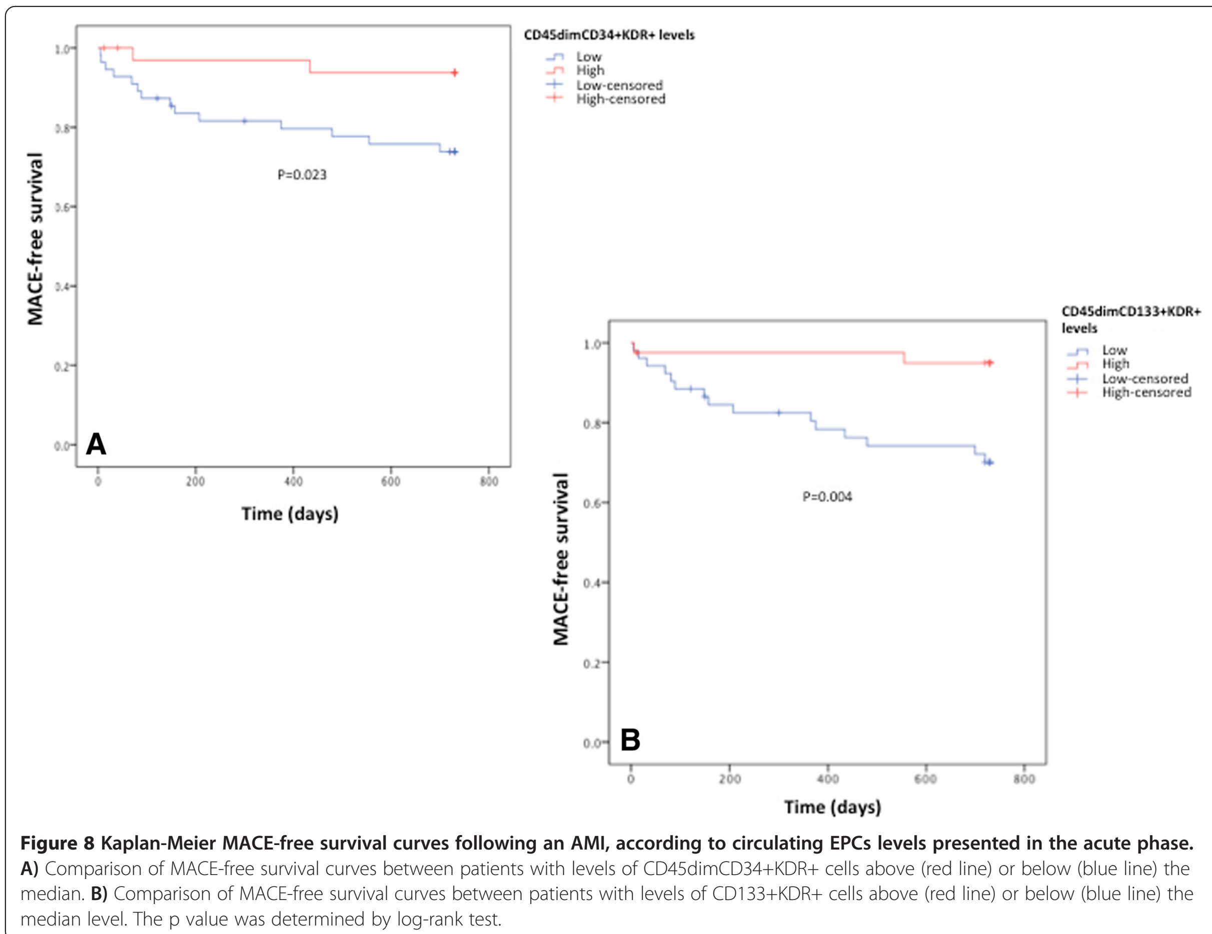

Figure 8 Kaplan-Meier MACE-free survival curves following an AMI, according to circulating EPCs levels presented in the acute phase. **A)** Comparison of MACE-free survival curves between patients with levels of CD45dimCD34+KDR+ cells above (red line) or below (blue line) the median. **B)** Comparison of MACE-free survival curves between patients with levels of CD133+KDR+ cells above (red line) or below (blue line) the median level. The p value was determined by log-rank test.

EPCs function [40-42]. More recently, Marfella et al. have demonstrated, in hyperglycemic patients with AMI, that EPCs levels increased after insulin infusion for intensive glycemic control [29]. Regarding oral antidiabetic drugs, several clinical studies have shown that PPAR-γ agonists, such as rosiglitazone and pioglitazone and also DPP-4 inhibitor sitagliptin increase EPCs levels and improve their function in diabetic patients [43-45]. However, little is known about the molecular mechanisms that regulate the beneficial effects of all these antidiabetic drugs over EPCs.

Importantly, evidence demonstrates that the degree of hyperglycemic control in diabetic patients is closely related to circulating EPCs levels [46,47]. However, despite the obvious interest to know the impact of chronic antidiabetic therapy on EPCs response of diabetic patients to an AMI, until now there have been no studies in the literature addressing this subject. Therefore, in the present work we have studied this issue and verified that, despite the longer DM duration and the worse glycemic control, insulin treated patients presented levels of CD45dimCD34+KDR+ EPCs that tended to approach that of nondiabetics. Conversely, CD45dimCD133+KDR+ EPCs and subpopulations coexpressing the CXCR4 receptor were not ameliorated by chronic insulin therapy, presenting the lowest levels in patients previously under insulin. Regarding oral antidiabetic drugs we were surprised to find no beneficial effect on EPCs levels, since these results differ from some published studies [43-45]. Notably, in accordance with the literature our results further demonstrated that levels of both CD45dimCD34+KDR+ and CD45dimCD133+KDR+ EPCs and even their subpopulations coexpressing the CXCR4 surface marker were inversely correlated with HbA1c, underscoring the importance of the glycemic control for EPCs response to an AMI. Taken together, these results suggest that insulin, but not oral antidiabetic drugs, may increase survival of circulating EPCs (denoted by the trend to the normalization of CD45dimCD34+KDR+ levels). So, it is tempting to speculate that the favorable clinical outcomes associated with glycemic control during AMI may be partly dependent on stimulation of EPCs-mediated neovascularization in the ischemic myocardium. However, even chronic insulin treatment

seemed unable to correct the characteristic dysfunction of diabetics EPCs (here illustrated by the decrease in CD45dimCD133+KDR+ EPCs, which may represent an impairment in mobilization from bone marrow, and reduction in CXCR4+ subpopulations, denoting a possible homing dysfunction). Yet, since patients under insulin therapy had the highest HBA1c levels, it is still unknown if with a better glycemic control chronic insulin therapy could reverse EPCs dysfunction of diabetic patients and completely normalize their response to an AMI. Altogether, our results suggest that chronic hyperglycemia and not diabetes *per se*, is the responsible for impaired EPCs response of diabetic patients to myocardial ischemia.

Limitations

The limitations of our study should be acknowledged: 1) the widespread interlaboratory variations in FACS methodology used to quantify circulating EPCs is still a problem. In this study we used a standardized protocol, which has demonstrated a high accuracy in the detection of different EPCs subpopulations with angiogenic properties and enable us to study the differentiation and commitment of these cells, from early precursors to more mature circulating EPCs [17,48]. However, we recognize that further standardization of EPCs definitions and FACS protocols would be important to better compare results between different groups; 2) the long list of exclusion criteria limited the enrollment of higher number of AMI patients in this study, resulting in a relatively small number of patients in each antidiabetic treatment group. Therefore, the data regarding the comparison of EPCs levels between the different antidiabetic treatment categories should be interpreted with caution because of the risk of error type II and further studies to explore how insulin therapy may interact and affect diabetic EPC numbers and function in patients with AMI, are obviously warranted; 3) since investigation of the molecular mechanisms regulating circulating EPCs levels in AMI diabetic patients was not under the scope of this study, the signaling pathways underlying the observed reduction in EPCs levels during the early phases of AMI in diabetic as compared to nondiabetic patients are unknown".

Conclusions

In summary, our data demonstrates that there is a progressive decrease in EPCs response to an AMI, according to the glycemic continuum, from NGM to pre-diabetes and finally DM, and that the exhaustion of the EPCs pool is influenced by the degree of glycemic control. Furthermore, it seems conceivable to use therapeutic interventions, such as insulin, to try to reverse the impaired response to an AMI of diabetics and possibly improve the dismal prognosis of these patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NA designed the study, contributed to clinical data acquisition and has written the first draft of the manuscript. RF contributed to data interpretation and critically revised the manuscript. AS helped to draft the manuscript and performed the statistical analysis. FS participated in the acquisition of clinical data. AL participated in interpretation of the data and helped to draft the manuscript. TC carried out the flow cytometry analysis and participated in interpretation of the data. AP contributed to the refinement of the research protocol, to the data analysis and interpretation, and to the development of the manuscript. GMP and LAP contributed to obtaining funding, and critically revised the manuscript. LG participated in the study design, contributed to obtaining funding and critically revised the manuscript. CFR conceived the study, participated in the data interpretation, oversaw the development of the manuscript and supervised the project. All authors read and approved the final manuscript.

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CHAPTER IV

Impact of prior chronic statin therapy and high intensity statin therapy at discharge on circulating endothelial progenitor cells levels in patients with acutemyocardial infarction: a prospective observational study

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PHARMACODYNAMICS

Impact of prior chronic statin therapy and high-intensity statin therapy at discharge on circulating endothelial progenitor cell levels in patients with acute myocardial infarction: a prospective observational study

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Abstract
Background Endothelial progenitor stem cells (EPCs) are mobilized to the peripheral circulation in response to myocardial ischemia, playing a crucial role in vascular repair. Statins have been shown to stimulate EPCs. However, neither the impact of previous statin therapy on EPC response of acute myocardial infarction (AMI) patients nor the effect of post-AMI high-intensity statin therapy on the evolution of circulating EPC levels has yet been addressed. Therefore, we aimed to compare circulating EPC levels between patients receiving long-term statin therapy before the AMI and statin-naive patients and to assess the impact of high-intensity statin therapy at discharge on the evolution of circulating EPCs post-AMI.
Methods This is a prospective observational study of 100 AMI patients. Circulating EPCs (CD45dimCD34+KDR+ cells) and their subpopulation coexpressing the homing marker CXCR4 were quantified by the high-performance flow cytometer FACSCanto II in whole blood, in two different moments: within the first 24 h of admission and 3 months post-AMI. Patients were followed up clinically for 2 years.
Results Patients previously treated with statins had significantly higher levels of EPCs coexpressing CXCR4 (1.9 ± 1.4 vs. 1.3 ± 1.0 cells/1,000,000 events, $p = 0.031$) than statin-naive patients. In addition, the subanalysis of diabetics ($N = 38$) also revealed that patients previously on statins had significantly greater numbers of both CD45dimCD34+KDR+CXCR4+ cells ($p = 0.024$) and CD45dimCD34+KDR+CD133+ cells ($p = 0.022$) than statin-naive patients. Regarding the evolution of EPC levels after the AMI, patients not on a high-intensity statin therapy at discharge had a significant reduction of CD45dimCD34+KDR+and CD45dimCD34+KDR+CXCR4+ cells from baseline to 3 months follow-up ($p = 0.031$ and $p = 0.005$, respectively). However, patients discharged on a high-intensity statin therapy maintained circulating levels of all EPC populations, presenting at 3 months of follow-up significantly higher EPC levels than patients not on an intensive statin therapy. Moreover, the high-intensity statin treatment group had significantly better clinical outcomes during the 2-year follow-up period than patients not discharged on a high-intensity statin therapy.
Conclusion Chronic statin therapy prior to an AMI strongly enhances the response of EPCs to myocardial ischemia, even in diabetic patients. Furthermore, high-intensity statin therapy after an AMI prevents the expected decrease of circulating EPC levels during follow-up. These results reinforce the importance of an early and intensive statin therapy in AMI patients.

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Keywords Endothelial progenitor cells · Statin · Myocardial infarction · Flow cytometry · Diabetes · Outcomes

Background

Endothelial progenitor cells (EPCs) were first described in 1997 by Ashara et al [1]. Since this initial report, the role of EPCs in neovascularization and tissue repair has been under intense investigation. It is now well accepted that EPCs are home to sites of ischemia, playing a crucial role in adult neovascularization and repair of damaged endothelium [2, 3]. In the clinical setting of an acute myocardial infarction (AMI), circulating EPC levels significantly increase and are rapidly recruited to the ischemic myocardium [4]. Mediating a protective effect on vascular repair, EPCs will improve blood supply to the ischemic penumbra, leading to decreased infarction area [5, 6]. Importantly, increased circulating levels of EPCs after an AMI have been associated with better clinical cardiovascular outcomes [7].

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase inhibitors), also known as statins, are the most effective class of drugs for lowering serum low-density lipoprotein (LDL) cholesterol concentrations [8] and are widely used in primary and secondary prevention of cardiovascular disease [9, 10]. Besides their well-known lipid-lowering action, statins exert several beneficial pleiotropic effects, which include stimulation of EPCs by improving their circulating numbers and functional activity [11–13].

The clinical benefits of statins after an AMI have been unequivocally demonstrated [14]. Given the well-established role of EPCs in vascular repair after ischemic injury, stimulation of EPCs by statins may contribute to the clinical benefit of HMG-CoA reductase inhibitors in patients with AMI. Furthermore, landmark trials have demonstrated that high-intensity statin therapy is superior to standard dose in the reduction of cardiovascular events after an AMI [15]. Therefore, according to current guidelines, statins should be started early during admission and should be given at high doses in all patients with AMI [16]. However, to date, there are no published studies evaluating the impact of previous statin therapy on circulating EPC levels in patients with an AMI. Moreover, the dose-dependent effects of a continuous statin therapy on EPCs in patients with AMI have not yet been analyzed.

This study was based on the hypothesis that patients chronically pretreated with statins present an improved response of circulating EPCs to an AMI compared to statin-naive patients and that a high-intensity statin therapy after discharge is better for the pool maintenance of EPCs than a less intensive statin therapy. Therefore, the aims of this study were (1) to compare circulating EPC levels between patients receiving long-term statin therapy and those not receiving statins before the AMI; (2) to evaluate the evolution of circulating EPCs, reassessing circulating EPC numbers 3 months later, to compare patients discharged on a high-intensity statin regimen or not.

Methods

Study population

This is a prospective observational study of consecutive patients hospitalized in a single coronary care unit (CCU) of a tertiary care teaching hospital, due to myocardial infarction, from 5 January 2009 to 23 September 2011. AMI was defined according to the latest European Society of Cardiology guidelines [16]. After exclusion criteria were applied, there were a total of 100 patients included in the study (65 % with ST segment elevation myocardial infarction—STEMI and 35 % with non-ST segment elevation myocardial infarction—NSTEMI). Patients were excluded if they were >80 years old and had clinical or biochemical evidence for the presence of concomitant inflammatory disease, known autoimmune or malignant diseases, severe peripheral arterial occlusive disease, deep vein thrombosis or pulmonary embolism, 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 <8.5 g/dl) or thrombocytopenia (<100,000/l), previous coronary bypass surgery, myocardial infarction (MI) within the preceding 2 months, cardiogenic shock, severe valvular disease or congenital heart disease, and comorbidities associated with a life expectancy less than 2 years. Regular use of nonsteroidal anti-inflammatory drugs or on anticoagulants, patients with pacemakers, implantable cardioverter defibrillators or resynchronization devices, and excessive alcohol consumption or illicit drug abuse that may influence EPC kinetics were also exclusion criteria. Patients were divided into two groups based on whether they were taking statins for ≥3 months before their admission (statin group—N=30) or were not taking any statin within the previous 3 months (no statin group—N=70). Patients were categorized by equivalent statin dosages on five levels (Table 1).

All patients received the standard therapy for the acute phase of MI that included acetylsalicylic acid (ASA), clopidogrel, and low molecular weight heparin, according to usual hospital practice. We also compared patients according to statin dose at discharge. For this comparison, patients were divided in two groups: (1) patients discharged on a high-intensity statin therapy (N=58) and (2) patients not discharged on a high-intensity statin therapy (N=42). The first group (high-intensity statin therapy) comprised patients receiving a statin equivalent dosage level 4 or 5 (Table 1). The second group (no high-intensity statin therapy) consisted of patients

Table 1 Statin equivalent dosage levels

	Atorvastatin (mg)	Fluvastatin (mg)	Lovastatin (mg)	Pravastatin (mg)	Rosuvastatin (mg)	Simvastatin (mg)
Dosage level 1	–	40	20	20	–	10
Dosage level 2	10	80	40	40	5	20
Dosage level 3	20	–	80	–	10	40
Dosage level 4	40	–	–	–	20	80
Dosage level 5	80	–	–	–	40	–

discharged on a low- to moderate-intensity statin therapy (N=38) and those discharged without statins (N=4).

Baseline demographic data, cardiovascular risk factors, and previous medications were recorded in all patients. Peripheral blood samples were collected from all patients at admission and in the next morning (day 1) after an overnight fast, to assess chemistry (including fasting glucose and glycosylated hemoglobin (HbA1C) and cardiac necrosis markers), total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides, and high sensitivity C-reactive protein (hs-CRP), according to standard hospital practice. Type 2 diabetes mellitus (DM) was defined according to the American Diabetes Association (ADA) criteria 2012 [17, 18].

This study was performed in accordance with the Declaration of Helsinki and was approved by the local Ethics Committee (Approval Number: HUC-23-08). Written informed consent was obtained from each patient.

Quantification of circulating EPCs by flow cytometry

EPCs were quantified by fluorescence-activated cell sorting (FACS), as previously described by our research group, at two different time points: (1) baseline (within the first 24 h of CCU admission) and (2) 3 months later [19]. Briefly, we have used a standardized protocol—the modified International Society for Hematotherapy and Graft Engineering (ISHAGE) sequential gating strategy—proposed by Schmidt-Lucke et al. [20] Whole blood samples were collected at baseline and 3 months after the AMI and were processed by FACS within 1 to 2 h of collection. Hence, 150 μl of whole blood was incubated with the following combination of antihuman monoclonal antibodies: 10 μl of anti-CD133 conjugated with allophycocyanin (APC) (Miltenyi Biotec), 5 μl of anti-CD45 conjugated with APC-H7 (Becton Dickinson), 10 μl of anti-KDR (also known as type 2 vascular endothelial growth factor receptor—VEGF-R2) conjugated with phycoerythrin (PE) (Sigma), 10 μl of anti-CD34 conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson), and 10 μl of anti-CD184 (also known as CXCR4) conjugated with PE-Cyanine 5 (PE-Cy5) (BD Pharmingen). Data acquisition was performed with a high-performance flow cytometer, a BDBioscience FACSCanto II, which can analyze with high resolution up to six different fluorescent markers from a large number of events. For the analysis, flow cytometry software Infinicyt 1.5 (Cytognos) was used. According to the used standardized protocol, human circulating EPCs were identified by a minimal antigenic profile that includes a marker of stemness/immaturity (CD34) plus a marker of endothelial commitment (KDR). CD45 staining was also performed to exclude leucocytes, as it has been previously demonstrated that only the fraction of CD45dim cells harbors the "true" circulating EPCs [21]. CXCR-4, the receptor for stromal cell-derived factor-1 (SDF-1), is a cell surface antigen expressed in EPCs, which plays a key role in their homing to sites of vascular injury [22]. Therefore, by analyzing the subpopulation of progenitor cells coexpressing CXCR4, we could study a functional parameter of EPCs. As isotype controls are known to mask rare cell populations, none were used in this analysis, and baseline fluorescence was determined using unstained cells [23]. Because EPCs are extremely rare events in peripheral blood, the total number of acquired events is increased to at least one million per sample, which is generally not needed for most applications of flow cytometry. Circulating EPCs were measured in triplicate from the same patients, revealing a very close correlation (r=0.87, p<0.0001). The same trained operator, who was blind to the clinical status of the patients, performed all the cytometric analysis throughout the study. Circulating EPC levels were expressed for one million cytometric events.

Three different populations of progenitor cells were analyzed: (1) CD45dimCD34+KDR+EPCs; (2) CD45dimCD34+KDR+CD133+ EPCs; and (3) CD45dimCD34+KDR+CXCR4+ EPCs. It has been reported that the coexpression of surface CD34, CD133, and KDR antigens identifies a population of early EPCs, whereas the lack of the CD133 antigen characterizes a more mature EPC population (CD45dimCD34+KDR+) [24].

Follow-up of patients for cardiovascular events

Patients were followed up for 24 months after discharge. We assessed cardiovascular medication use (including statin dosages) at 24 months follow-up in patients alive. The following cardiovascular events were recorded: cardiovascular death, nonfatal stroke or transient ischemic attack, reinfarction, unstable angina, and rehospitalization for unstable angina or

heart failure. We also analyzed the combined endpoint of cardiovascular death, rehospitalization for ACS, and unplanned percutaneous coronary intervention (PCI)–major adverse cardiac events (MACE). Cardiovascular death was defined as death due to a MI or stroke or documented sudden cardiac death. For patients experiencing more than one acute event, only the first event was considered in the analysis.

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 range for parametric data or median±interquartile for nonparametric data. Categorical data is expressed as counts and percentages.

For comparison of continuous data between groups, unpaired Student's *t* test was used when variables were normally distributed and nonparametric Mann–Whitney test was employed for variables without a normal distribution. The evolution of circulating EPC numbers from baseline to 3 months of follow-up was analyzed with the paired Student's *t* test, by comparing numbers of EPCs in the two time points, for each patient. Categorical variables were compared with the chi-square test or with Fisher exact test as appropriate. The relationship between variables was assessed using Pearson's or Spearman's correlation coefficient, whichever is appropriate. Multivariate logistic regression analysis with the enter method was performed to identify independent predictors of circulating EPC levels. For all analyses, a two-sided value of $p<0.05$ was considered statistically significant.

Results

Characteristics of the study population

Before hospital admission, 70 patients were statin-naive and 30 patients were chronically treated with statins (36.7 % (11 patients) with atorvastatin, 33.3 % (10 patients) with simvastatin, 10 % (3 patients) with fluvastatin, 10 % more with rosuvastatin, and the other 10 % were on pravastatin). Analyzing statin equivalent doses, 20 patients (66.7 % of statin pretreated patients) were medicated with a low statin dosage (5 patients on dosage level 1 and 15 patients on dosage level 2) and 10 patients (33.3 %) were on an intermediate dosage of statin (all of them on a dosage level 3).

Regarding baseline clinical characteristics, patients previously treated with statins were significantly older, had more frequently a previous MI, and therefore received more beta-blockers, ASA, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs) than statin-naive patients. Additionally, the diagnosis of STEMI was less frequent in patients treated chronically with statins. However, there were no other significant differences in cardiovascular risk factors or baseline laboratorial parameters between groups, with the exception of the expected difference in dyslipidemia and cholesterol levels (Table 2). Not negligible was the observation where statin-treated patients tended to present lower levels of high sensitivity C-reactive protein (hs-CRP) on admission than statin-naive patients (Table 2).

Chronic statin treatment was associated with significantly higher levels of circulating EPCs in the acute phase of a MI

Patients previously treated with statins had significantly higher levels of the subpopulation of EPCs coexpressing CXCR4 than statin-naive patients (1.9±1.4 vs. 1.3±1.0 cells/1,000,000 events, p=0.031). We have not found significant differences between groups either in the levels of CD45dimCD34+KDR+EPCs or in the levels of the more immature population CD45dimCD34+KDR+CD133+ EPC levels (5.2±3.5 in statin-treated patients vs. 4.9± 3.5 in naive patients, p=0.820 and 1.4±1.2 in the statin group vs. 1.2±1.1 cells/1,000,000 events in the statin-naive group, p=0.519) (Fig. 1). Of note, there were no significant differences in the levels of any EPC subpopulation according to prior use or not of ASA, β-blockers, ACE inhibitors, or ARBs. Furthermore, multivariate analysis revealed that chronic statin therapy prior to the AMI remained an important determinant of CD45dimCD34+KDR+CXCR4+ EPC levels even after adjustment for potential confounding variables (Fig. 2).

No significant correlations were found between EPC numbers and plasma levels of cholesterol or cardiac necrosis markers. There were 38 patients with DM, and of these, 31.6 % (12 patients) were previously treated with a statin. Considering the subanalysis of diabetic patients, we verified that patients chronically treated with statins also presented higher levels of circulating EPCs. In fact, diabetic patients previously on statin treatment had significantly increased numbers of both CD45dimCD34+KDR+CXCR4+ cells (1.3±0.7 vs. 0.8±0.5 cells/1,000,000 events in statin-naive diabetics, p=0.024) and CD45dimCD34+KDR+CD133+ cells (2.3±1.9 vs. 0.9±0.8 cells/1,000,000 events in diabetics not previously treated with statins, p=0.022) (Fig. 3).

Importantly, admission hs-CRP levels were significantly lower in diabetics previously on statins when compared with statin-naive diabetics (0.25±0.14 vs. 0.45±0.34 mg/dl, respectively, p=0.035) (Fig. 4). Additionally, we found a significant inverse correlation between admission hs-CRP levels and CD45dimCD34+KDR+CD133+ numbers in diabetics (r=−0.419, p=0.026).

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Table 2 Comparison of clinical characteristics between patients chronically on statin therapy and statin-naive patients

	Previous statin (*N*=30)	No previous statin (*N*=70)	*p* value
Age (years)[a]	62.7±8.3	58.4±11.7	0.037
Male gender (%)	83.3	92.9	0.146
BMI (kg/m^2)[a]	28.1±2.9	28.5±6.2	0.524
Previous MI (%)	36.7	4.3	<0.001
Type of AMI			
STEMI vs. NSTEMI (%)	48.3/51.7	71.4/28.6	0.038
Cardiovascular risk factors			
Type 2 diabetes mellitus (%)	60.0	62.9	0.825
Hypertension (%)	70.0	65.7	0.817
Smoking habits (%)	60.0	54.3	0.598
Family history (%)	37.9	32.9	0.648
Hyperlipidemia (%)	96.7	70.0	0.003
Physical inactivity (%)	60.0	57.1	0.828
Previous cardiovascular drugs			
ASA (%)	60.0	14.3	<0.001
ACEI (%)	36.7	15.7	0.033
ARB (%)	40.0	11.4	0.002
Beta-blockers (%)	36.7	4.3	<0.001
Baseline laboratory			
Admission troponin I (μg/l)[b]	19.2±56.2	15.4±49.2	0.735
Peak troponin I (μg/l)[b]	55.6±79.5	55.2±64.2	0.872
HbA1C (%)[b]	6.0±0.9	6.2±1.1	0.607
Admission glycemia (mg/dl)[b]	136.7±44.2	149.0±57.3	0.248
First fasting glycemia (mg/dl)[b]	121.6±29.3	124.2±41.7	0.759
Total cholesterol (mg/dl)[a]	183.4±61.6	205.7±56.2	0.024
LDL cholesterol (mg/dl)[a]	117.2±46.3	139.4±43.3	0.011
HDL cholesterol (mg/dl)[b]	42.8±11.8	40.0±10.6	0.275
Triglycerides (mg/dl)[b]	188.9±191.4	182.4±138.3	0.634
Uric acid (mg/dl)[a]	5.9±1.3	5.8±1.4	0.793
Admission hs-CRP (mg/dl)[a]	0.7±0.8	1.1±1.6	0.068
LVEF (%)[a]	50.4±9.7	51.5±10.9	0.674

ACEI angiotensin-converting enzyme inhibitors, *AMI* acute myocardial infarction, *ARB* angiotensin II receptor blockers, *ASA* acetylsalicylic acid, *hs-CRP* high sensitivity C-reactive protein, *HbA1C* hemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *LVEF* left ventricular ejection fraction, *MI* myocardial infarction, *STEMI* ST elevation myocardial infarction, *NSTEMI* non-ST elevation myocardial infarction

[a] Mean±SD

[b] Median±interquartile range

Evolution of circulating EPC levels after acute myocardial infarction

Ninety-six percent of patients received statin therapy at discharge. Regarding dosages, 58 % of patients were discharged on high-intensity statin therapy (55 patients on a dosage level 4 and 3 patients on a level 5). Between diabetic patients, 50 % were prescribed high-statin dose at discharge. However, only 50 % of all patients discharged on high-intensity statin therapy maintained the same intensity of treatment at 2-year follow-up. Unsurprisingly, almost all patients (91 %) had an upgrading of statin dose from pre-AMI phase to hospital discharge. Among patients discharged on high-intensity statin therapy, only 14 (24 %) were already on statins before the AMI and all of them underwent intensification of statin treatment. In the group of patients not discharged on high-intensity statin therapy (42 patients), 34 patients (81 %) received statin on a dosage level 3 at discharge, 4 patients (9.5 %) did not receive any statin, and the remaining 4 patients were discharged with statin therapy on dosage level 2.

There were no significant differences in the proportion of patients receiving ACE inhibitors, ASA, clopidogrel, or β-blockers at discharge between the high-intensity statin therapy group and the group of patients not discharged on high-intensity statin therapy (Table 3). Furthermore, both groups were comparable regarding clinical characteristics, except for the fact that patients discharged on a high-intensity statin therapy were younger and had better left ventricular systolic function than patients not on a high-intensity statin therapy at discharge (Table 3).

To investigate the effect of high-intensity statin therapy on circulating EPC evolution after the AMI, their levels were

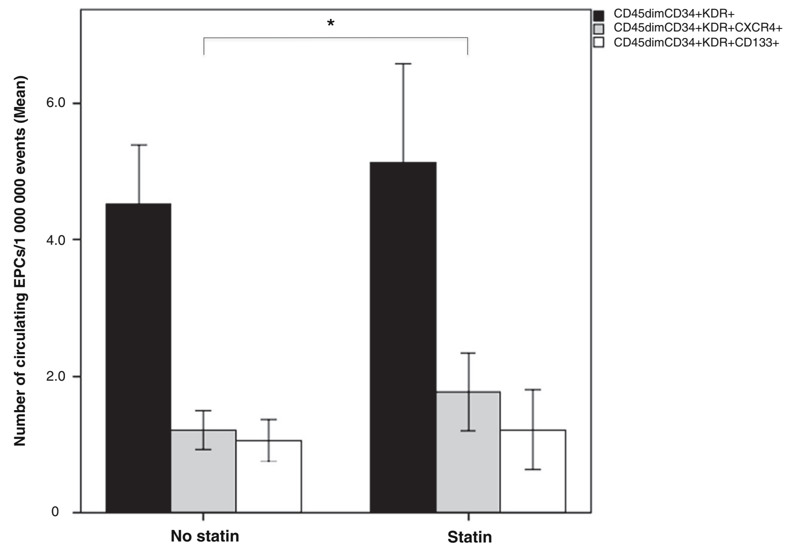

Fig. 1 Comparison of circulating EPC levels between patients under chronic statin treatment and statin-naive patients. The *bar charts* show mean±standard deviation of circulating EPC levels of patients previously treated with statins by comparison with statin-naive patients. *Black bars*=CD45dimCD34+KDR+ EPCs; *gray bars*=fraction of EPCs coexpressing the receptor CXCR4; *white bars*=fraction of EPCs coexpressing CD133. The comparison between the two groups was performed by Student's *t* test. An *asterisk* (*) indicates $p<0.05$. *EPCs* endothelial progenitor cells

reassessed at 3 months of follow-up. We found significant differences in the evolution of circulating EPCs between patients discharged on high-intensity statin therapy or not. Patients not on high-intensity statin therapy at discharge showed a significant reduction of CD45dimCD34+KDR+and CD45dimCD34+KDR+CXCR4+ cells from baseline to the post-AMI period ($p=0.031$ and $p=0.005$, respectively) (Table 4). However, patients discharged on a high-intensity statin regimen did not experience reduction in circulating levels of any EPC population (Table 4). Additionally, at 3 months follow-up, patients on high-intensity statin therapy showed significantly higher levels of circulating CD45dimCD34+KDR+and CD45dimCD34+KDR+CXCR4+ cells than patients without this intensive therapy (Table 4 and Fig. 5). At multivariate analysis, high-intensity statin therapy at discharge was an independent predictor of higher circulating levels of CD45dimCD34+KDR+EPC, at 3 months follow-up (hazard ratio 28.7; 95 % CI 1.38–59.8, $p=0.030$). However, multivariate analysis did not confirm high-intensity statin therapy as an independent predictor of circulating CD45dimCD34+KDR+CXCR4+ EPC levels.

Post-AMI clinical outcomes according to intensity of the statin therapy at discharge

No patient was lost to follow-up during the 24-month period. By comparing cardiovascular outcomes according to the

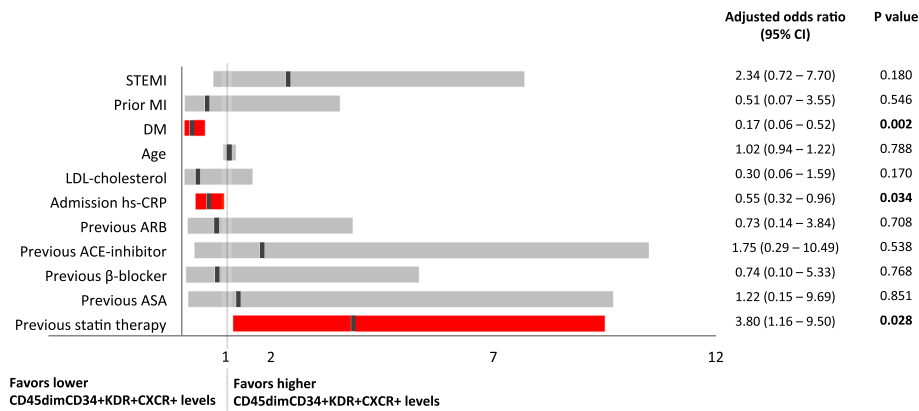

Fig. 2 Multivariate analysis (logistic regression) to predict higher levels of CD45dimCD34+KDR+CXCR4+ EPCs during the acute phase of myocardial infarction. *Red bars* represent the independent predictors of EPC levels. Lower CD45dimCD34+KDR+CXCR4+ EPC levels refer to levels below the median and higher CD45dimCD34+KDR+CXCR4+ EPC levels refer to levels above the median for circulating CD45dimCD34+KDR+CXCR4+ cells. Variables introduced in the model were those with significant differences in the univariate analysis between patients previously on statins and statin-naive patients and variables considered clinically relevant for the analysis. *ACE inhibitor* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker, *ASA* acetylsalicylic acid, *CI* confidence interval, *DM* type 2 diabetes mellitus, *hs-CRP* high sensitivity C-reactive protein, *MI* myocardial infarction, *STEMI* ST segment elevation myocardial infarction

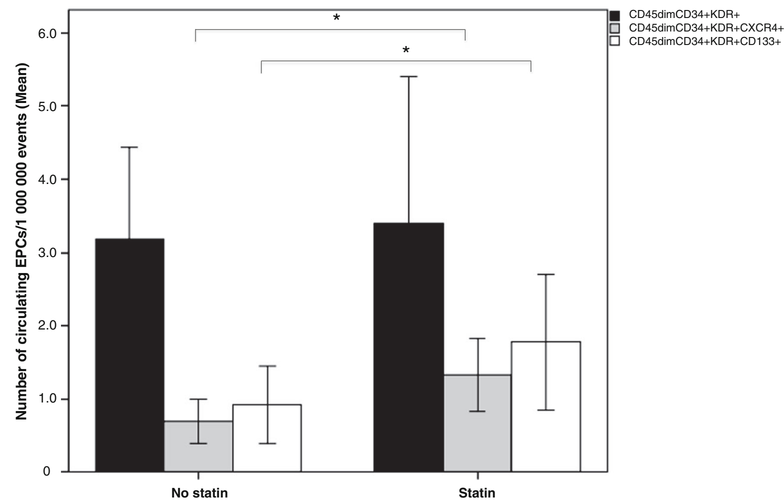

Fig. 3 Comparison of circulating EPC levels between diabetic patients previously on statin and statin-naive diabetics. The *bar charts* show mean±standard deviation of circulating EPC levels of diabetics previously treated with statins by comparison with statin-naive diabetics. *Black bars*=CD45dimCD34+KDR+EPCs; *gray bars*=fraction of EPCs coexpressing the receptor CXCR4; *white bars*=fraction of EPCs coexpressing CD133. The comparison between the two groups was performed by Student's *t* test. An *asterisk* (*) indicates $p<0.05$. *EPCs* endothelial progenitor cells

intensity of statin therapy at discharge, we verified that patients on a high-intensity statin therapy tended to present a better evolution than patients not on a high-intensity statin therapy. In fact, there were no cardiovascular deaths during the 2-year follow-up period in the more intensive treatment group by comparison with a 7.1 % cardiovascular mortality rate in patients not discharged on a high-statin dose ($p<0.05$). Moreover, ischemic stroke, reinfarction, and the composite event MACE also tended to occur less frequently in patients on a high-intensity statin regimen at discharge (Table 5). There were no significant differences in the rates of unstable angina, unplanned PCI, or rehospitalization for unstable angina or heart failure between the two treatment groups (Table 5).

Discussion

The major observation of this study is that chronic statin therapy prior to an AMI enhances the response of EPCs to myocardial ischemia, inducing a significant increase in circulating levels of EPCs coexpressing the homing receptor

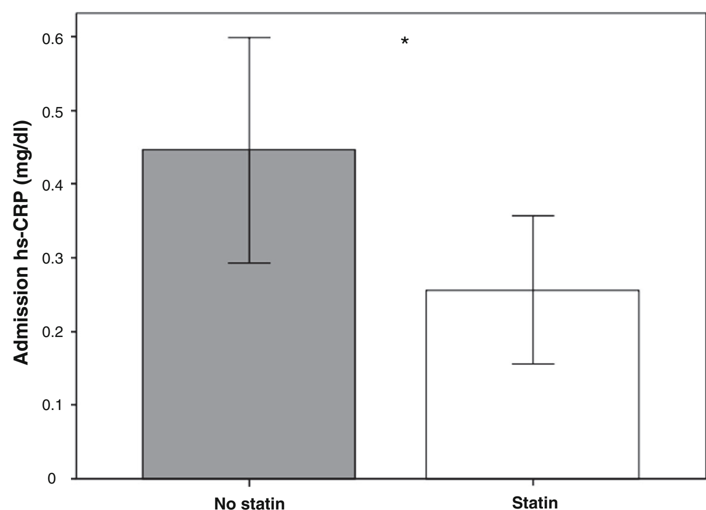

Fig. 4 Comparison of admission levels of C-reactive protein between diabetics on statins and statin-naive diabetics. The *bar charts* represent the differences between diabetics not previously on statins (*gray bar*) and diabetics previously treated with statins (*white bar*) in regard to mean levels of admission of high sensitivity C-reactive protein (hs-CRP). *Error bars* represent the standard deviation (Student's *t* test, *$p<0.05$)

Table 3 Comparison of clinical characteristics between patients discharged on a high-intensity statin therapy or not

	High-intensity statin therapy (*N*=58)	No high-intensity statin therapy (*N*=42)	*p* value
Age (years)[a]	57.8±9.9	62.1±12.0	0.049
Male gender (%)	89.7	90.5	0.870
BMI (kg/m^2)[a]	28.9±5.8	27.7±5.0	0.317
Previous MI (%)	12.1	19.0	0.229
Type of AMI			
STEMI vs. NSTEMI (%)	79.3/20.7	43.9/56.1	<0.001
Cardiovascular risk factors			
Type 2 diabetes mellitus (%)	65.5	57.1	0.333
Hypertension (%)	65.5	69.0	0.666
Smoking habits (%)	60.3	50.0	0.258
Family history (%)	34.5	34.1	0.923
Hyperlipidemia (%)	77.6	78.6	0.870
Physical inactivity (%)	55.2	64.3	0.323
Cardiovascular drugs at discharge			
ASA (%)	93.1	100.0	0.181
ACEI (%)	74.1	82.1	0.338
Clopidogrel (%)	93.1	87.2	0.264
Beta-blockers (%)	93.1	89.7	0.355
Baseline laboratory			
Admission troponin I (μg/l)[b]	14.5±34.9	19.4±68.0	0.639
Peak troponin I (μg/l)[b]	52.6±55.2	61.5±84.4	0.526
HbA1C (%)[b]	6.1±1.0	6.1±1.1	0.863
Admission glycemia (mg/dl)[b]	145.8±54.8	142.0±50.7	0.732
First fasting glycemia (mg/dl)[b]	120.6±36.9	125.6±39.2	0.522
Total cholesterol (mg/dl)[a]	201.1±60.8	197.8±55.7	0.786
LDL cholesterol (mg/dl)[a]	134.9±48.4	131.2±40.9	0.690
HDL cholesterol (mg/dl)[b]	40.2±10.5	42.1±11.6	0.396
Triglycerides (mg/dl)[b]	189.5±158.1	175.1±153.5	0.657
Uric acid (mg/dl)[a]	5.9±1.2	5.8±1.6	0.609
Admission hs-CRP (mg/dl)[a]	0.7±1.0	0.9±1.4	0.514
LVEF (%)[a]	53.0±8.9	48.2±12.5	0.042

ACEI angiotensin-converting enzyme inhibitors, *AMI* acute myocardial infarction, *ARB* angiotensin II receptor blockers, *ASA* acetylsalicylic acid, *hs-CRP* high sensitivity C-reactive protein, *HbA1C* hemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *LVEF* left ventricular ejection fraction, *MI* myocardial infarction, *STEMI* ST elevation myocardial infarction, *NSTEMI* non-ST elevation myocardial infarction

[a] Mean±SD

[b] Median±interquartile range

CXCR4+. To the best of our knowledge, this is the first study to investigate the effects of chronic statin pretreatment on circulating EPC levels expressed in the early phases of an AMI. Another novel finding is that high-intensity statin therapy prevents the reduction of circulating EPC numbers expected to occur during the post-MI period.

AMI has always been closely associated with the concept of irreversible tissue damage followed by scar replacement

Table 4 Evolution of circulating EPC levels from baseline to 3 months follow-up

		Baseline	3-month FU	*p* value
CD45dimCD34+KDR+cells/1,000,000 events	High-intensity statin therapy	5.4±3.7	5.5±2.1	0.875
	No high-intensity statin therapy	4.6±3.2	3.2±1.9	0.032
CD45dimCD34+KDR+CXCR4+ cells/1,000,000 events	High-intensity statin therapy	1.4±1.0	1.6±1.2	0.485
	No high-intensity statin therapy	1.5±1.1	0.7±0.3	0.005
CD45dimCD34+KDR+CD133+ cells/1,000,000 events	High-intensity statin therapy	1.3±1.0	1.5±1.2	0.493
	No high-intensity statin therapy	1.3±1.2	1.1±0.8	0.760

FU follow-up

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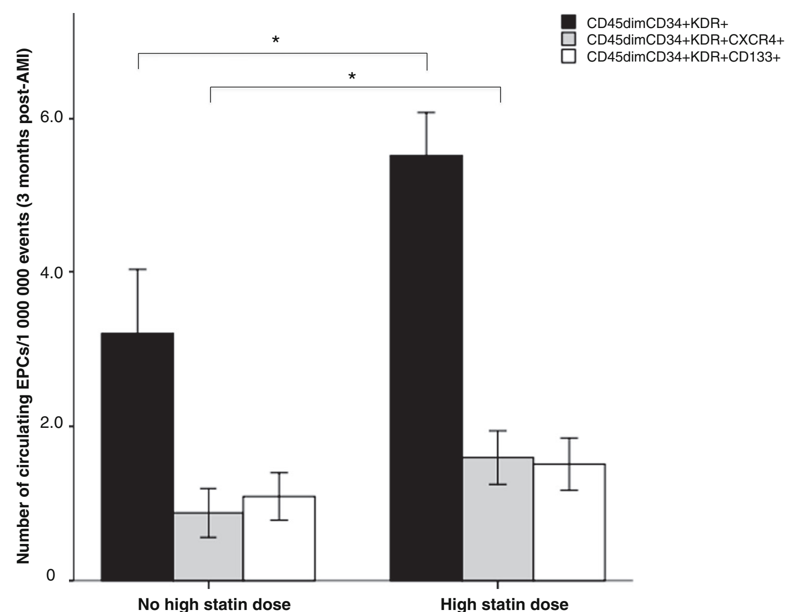

Fig. 5 Comparison of circulating EPC levels at 3 months follow-up, between patients discharged or not on high-intensity statin therapy. The *bar charts* show mean±standard deviation of circulating EPC levels of patients discharged on high-intensity statin therapy (high-statin dose) by comparison with patients not discharged on a high-intensity regimen (no high-statin dose). *Black bars*=CD45dimCD34+ KDR+EPCs; *gray bars*=fraction of EPCs coexpressing the receptor CXCR4; *white bars*= fraction of EPCs coexpressing CD133. The comparison between the two groups was performed by Student's *t* test. An *asterisk* (*) indicates $p<0.05$. *EPCs* endothelial progenitor cells

[4]. Nevertheless, in the last decade, data documenting myocardial tissue regeneration have opened the door to pharmacological modulation of stem cells and cell-based therapy for myocardial repair in patients with AMI [25–28]. Postnatal neovascularization was originally considered to occur exclusively through the mechanism of angiogenesis, which consists of neovessel formation, operated by in situ proliferation and migration of preexisting endothelial cells [29]. However, the discovery of EPCs resulted in the addition of a new mechanism, vasculogenesis, which involves new vessel formation by in situ incorporation and differentiation of bone marrow-derived EPCs into mature endothelial cells [30, 31]. In response to myocardial ischemia, local tissue vascular endothelial growth factor (VEGF) release initiates the vasculogenic cascade by activating EPCs from their quiescent state in the bone marrow, stimulating then their mobilization into circulation and homing to areas of needed neovascularization [32]. A similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF) and stroma-derived factor-1 (SDF-1) [33, 34]. The SDF-1/CXCR-4 axis seems particularly important in the homing, chemotaxis, engraftment, and retention of EPCs in the ischemic myocardium [35]. Importantly, it has been previously demonstrated that circulating EPCs markedly increase in the early phase of AMI and normalize to baseline levels within 2 months [4]. On the other hand, there is a strong body of evidence suggesting that mobilization of EPCs may be induced, not only by natural hematopoietic or angiogenic stimulants, but also by pharmacological agents. In fact, several published studies have shown that one of the pleiotropic effects of statins is the stimulation of circulating numbers and functional activity of EPCs [11–13].

In this study, chronic statin pretreatment was associated with an approximately 1.5-fold increase in the circulating levels of CD45dimCD34+KDR+CXCR4+ EPCs presented in the very early phase of an AMI. CXCR4, a chemokine receptor, has been shown to play a pivotal role in regulating EPC engraftment and function in vascular remodeling and neovascularization [36, 37]. Therefore, by analyzing the expression of CXCR4 on EPCs, it is possible to study a functional parameter—homing of EPCs to target ischemic sites. Since patients pretreated with statins showed significantly higher levels of the EPCs subset CXCR4+ than statin-naive patients, we can speculate that statin therapy improves the homing of EPCs to foci of ischemia or vascular injury. These results are in accordance with a previous study by Walter et al. who have demonstrated that statins induce adhesiveness of cultured human EPCs and accelerate reendothelialization by upregulation of the integrin subunits α5, β1, αv, and β5 [35]. They are also in good agreement with data from Vasa et al., showing that statin therapy increases circulating EPC numbers and enhances functional activity in patients with stable coronary artery disease (CAD) [11]. Moreover, Dimmeler et al. have demonstrated that statins increase EPC counts in vitro via stimulation of the PI 3-kinase/Akt signaling pathway [38]. The present study further extends these findings by showing that even diabetic patients may benefit from the positive effects of prior statin therapy on the response of EPCs to an AMI. In fact, diabetics previously treated with statin presented significantly higher levels of both CD45dimCD34+KDR+

Table 5 Comparison of clinical outcomes during the 2-year follow-up period, between patients discharged on high-intensity statin therapy or not

	High-intensity statin therapy at discharge	No high-intensity statin therapy at discharge	*p* value
CV mortality (%)	0.0	7.1	0.040
Nonfatal stroke/TIA (%)	0.0	4.8	0.096
Reinfarction (%)	0.0	4.9	0.092
MACE (%)	12.3	24.4	0.099
UA (%)	8.8	9.8	0.868
Unplanned PCI (%)	12.3	17.1	0.504
Rehospitalization for UA or HF	14.0	19.0	0.503

CV cardiovascular, *MACE* major adverse cardiac events, *PCI* percutaneous coronary intervention, *TIA* transient ischemic attack, *UA* unstable angina

CXCR4+ and CD45dimCD34+KDR+CD133+ cell subsets. This is of particular importance since diabetes is associated with worse clinical outcomes after an AMI and this dismal prognosis may be explained by the well-characterized reduced numbers and impaired function of EPCs in diabetics [7, 39, 40]. The mechanisms contributing to the reduction of circulating EPCs in diabetes are presently not fully understood; however, it may reflect a shortened peripheral survival of these cells, and/or a poor mobilization of EPCs from bone marrow to the peripheral circulation [40]. In our work, circulating levels of the more immature EPC population (coexpressing the stemness marker CD133) were significantly increased in diabetics pretreated with statins. This finding suggests that statin therapy is able to stimulate bone marrow mobilization of EPCs even in the clinical setting of DM. Furthermore, the increased levels of the CXCR4+ subpopulation in previously statin-treated diabetics also suggest a benefit of statins over homing of EPCs even in diabetes. These results may have important clinical implications, since they indicate that impaired functional activity of diabetic EPCs can be pharmacologically improved and, therefore, does not limit the feasibility of autologous cell-based therapy in this high cardiovascular risk population.

Importantly, this study revealed that diabetics previously on statin therapy presented half the level of admission hs-CRP compared to statin-naive diabetics. Moreover, circulating levels of the more immature subset of EPCs (CD45dimCD34+KDR+CD133+ cells) showed a moderate inverse correlation with hs-CRP in the diabetic subgroup. Diabetes is associated with continuous, low-level inflammation, leading to an increase in the prototype marker of inflammation, hs-CRP levels [41]. Notably, previous in vitro studies have demonstrated that hs-CRP, at concentrations known to predict adverse vascular outcomes, directly impairs EPC levels and function [42, 43]. Therefore, it is easy to understand the inverse relationship between hs-CRP and EPC levels in diabetics found in the present study.

Statins exhibit a wide spectrum of pleiotropic effects, which include among others immunomodulatory and anti-inflammatory properties, improvement of endothelial dysfunction, antioxidant effects, increase in nitric oxide bioavailability, protection against ischemia damage, inhibition of cardiac hypertrophy, slight antithrombotic property, and stabilization of atherosclerotic plaques [44]. There is compelling evidence that statins, as anti-inflammatory agents, may decrease plasma hs-CRP levels in patients with AMI and attenuate the effect of inflammation on risk of cardiovascular events [45–47]. Our results are in accordance with the previously demonstrated favorable effect of statins on the inflammatory pathways, since patients previously on statins showed lower admission levels of hs-CRP.

Interestingly, patients on a high-intensive statin therapy, as opposed to patients not discharged on a high-intensity statin regimen, did not undergo the expected decrease in circulating EPCs post-AMI, presenting significantly higher levels of EPCs at 3 months follow-up. This suggests that high-intensive statin therapy at discharge can counteract the expected decline in EPC levels after the AMI, because statins are a very strong stimulus for EPC recruitment that they parallel the stimulation mediated by myocardial ischemia. However, since patients discharged on high-intensity statin therapy were younger, had more frequent STEMI, and had a better left ventricular systolic function, we cannot rule out a possible contribution of clinical characteristics for the lower decline in circulating EPC levels. Similar to our data, Leone et al. have observed that high-intensity statin treatment was associated with higher EPC numbers at follow-up as compared to standard treatment in STEMI patients [48]. Additionally, Satoh et al. have reported that intensive lipid-lowering therapy increases EPC numbers and prevents telomere-shortening-induced senescence of EPCs compared with moderate-statin doses in patients with CAD [49].

It is well recognized that intensive statin treatment reduces the risk of recurrence of cardiovascular events in patients with MI [15]. Unfortunately, in the present observational study, despite the almost universal prescription of statins at discharge (96 %), only 58 % of patients were discharged on a high-statin dose. However, this suboptimal prescription rate is higher than that reported in previous “real-world” clinical studies and registries [50–52]. Moreover, despite the fact that diabetic patients represent a high-risk AMI cohort, with nearly double the rate of mortality and reinfarction compared with

nondiabetic patients, there is an even lower rate of intensive statin prescription at discharge in these patients. In a large, multicenter registry of 1,300 diabetic patients with AMI, only 22 % were prescribed intensive statin therapy at hospital discharge [51]. Reduced numbers of circulating EPCs have been associated with the occurrence of cardiovascular events in patients with CAD [53]. Therefore, one might speculate that statin-mediated stimulation of circulating EPCs plays a pivotal role in improving prognosis after AMI. Of note, in the present study, patients discharged on a high-intensity statin therapy had considerably better clinical outcomes post-AMI. This could be related to the statins putative effect on EPCs, since patients discharged on high-intensity statin therapy presented significantly higher levels of circulating CD45dimCD34+ KDR+and CD45dimCD34+KDR+CXCR4+ cells 3 months after the AMI compared with patients not discharged on a high-intensity statin therapy. However, these results are hypothesis-generating and require further investigation.

Limitations

The present study has some limitations that should be considered in the interpretation of results. (1) Regarding high-intensity statin therapy and its possible benefit on cardiovascular outcomes, due to the low incidence of cardiovascular events in our study cohort during the 2-year follow-up period, multivariate analysis to address the interaction of potential confounding factors could not be performed. (2) The positive effects of the intensive statin therapy over EPCs seem to be independent of other cardiovascular drugs. In fact, there were no significant differences in the proportion of patients receiving other cardiovascular drugs at discharge between both groups (patients discharged on high-intensity statin therapy or not). However, it should be noted that patients on a high-intensity statin therapy were significantly younger and had a better systolic function than patients not discharged on high-intensity statin therapy. This could contribute to the differences in outcomes observed between groups during follow-up. (3) We did not know whether the positive effects of statins over circulating EPCs observed in our study result in their participation in neovascularization after AMI, their actual role in myocardial regeneration. (4) Because this was a single-center observational study with many exclusion criteria, the subanalysis of diabetics had a small number of patients, and these results need further confirmation in larger cohorts. (5) Finally, the present study does not address the mechanisms by which statins lead to stimulation of EPCs. Although several previous experimental studies have studied the mechanisms responsible for the beneficial effects of statins on EPCs, further clinical studies are required to understand the mechanisms through which EPCs are mobilized after an AMI and to identify the exact role of EPCs in this setting.

Conclusions

In conclusion, our results indicate that chronic statin therapy potently enhances the response of EPCs to myocardial ischemia, even in diabetic patients. Furthermore, the present study demonstrates for the first time that high-intensity statin therapy prevents the expected decrease of circulating EPC levels to baseline values after an AMI. These findings have important therapeutic implications, as they reinforce the importance of early and intensive statin therapy after an AMI.

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CHAPTER V

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

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Endothelial progenitor cells in diabetic patients with myocardial infarction – Can statins improve their function?

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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. In addition, 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.
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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). In addition, 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).

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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. In addition, 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 to 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 over 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 <8.5 g/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 of 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, 5×10^6 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 1×10^6 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 et al. 2006b, with minor modifications. Briefly, MNCs were plated on 6-well fibronectin-coated plates (Biocoat BD) at a density of 5×10^6 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)-labeled acetylated low-density lipoprotein (acLDL; Molecular Probes) (which labels endothelial cells, via receptor-mediated endocytosis) and fluorescein isothiocyanate (FITC)-labeled 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 37 °C, for 1 h, washed thrice with PBS and then fixed with 2% paraformaldehyde for 10 min.

Cells were then incubated with UEA-1 (40 μg/ml) at 4°C, 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 4 °C 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 given 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 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 5×10^6 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 1.45s 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, 3×10^5 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 analyzed 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.

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.

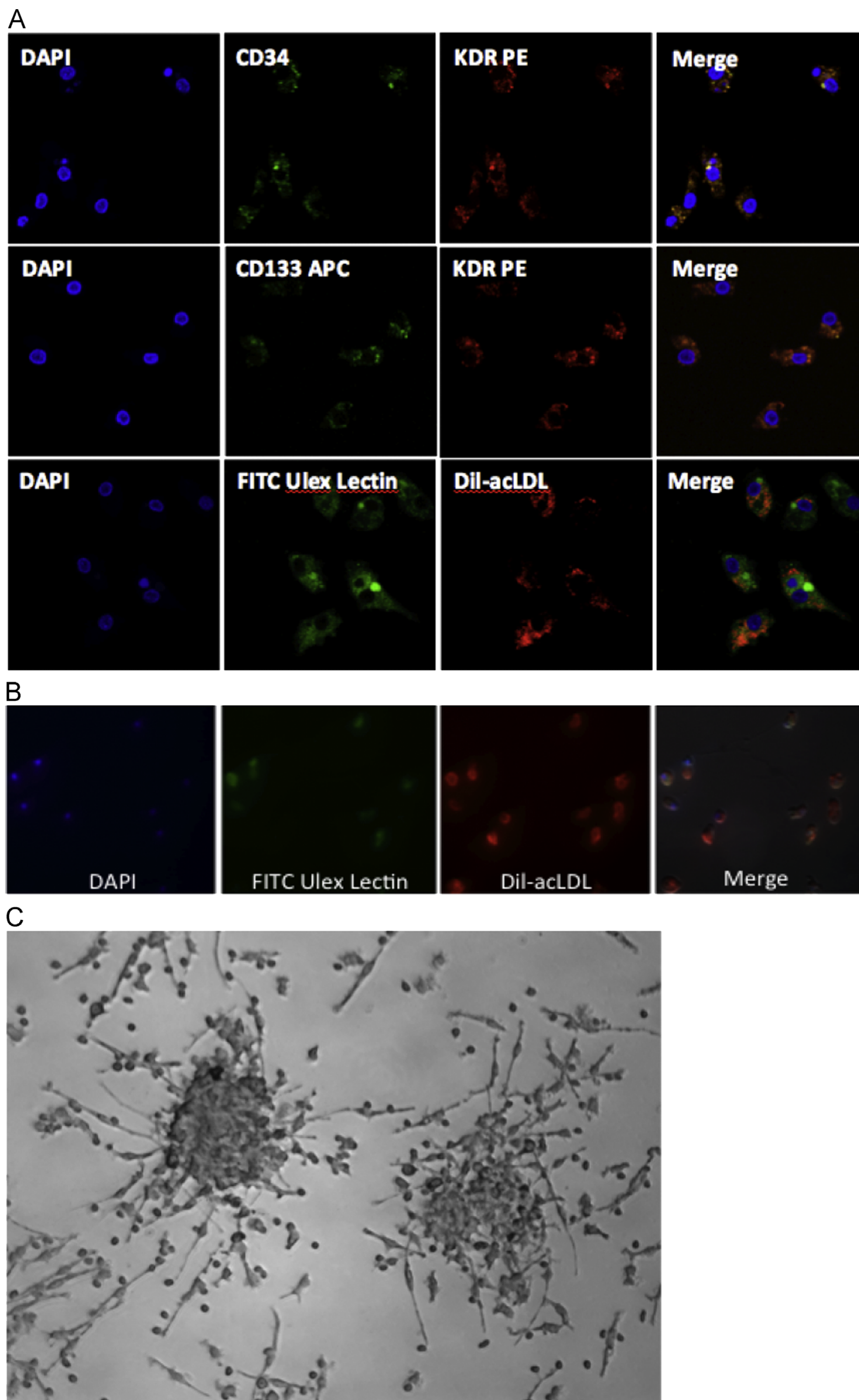

Fig. 1. Characterization of early EPCs at day 7 (A) and late EPCs at day 15 (B). Representative images are shown for cells derived from a diabetic STEMI patient cultured under control conditions. Nuclear stain DAPI (blue) was used to identify cells by fluorescence microscopy. Images (400 $\times$) illustrate that all adherent cells were positively labeled with DiI-acetylated LDL, (DiI-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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 to 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 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. 1A 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. In addition, 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. 1C). These cells showed the characteristic Dil-Ac-LDL uptake and UEA-1 lectin binding.

Under control conditions, the number of cell colonies generated from 5×10^6 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).

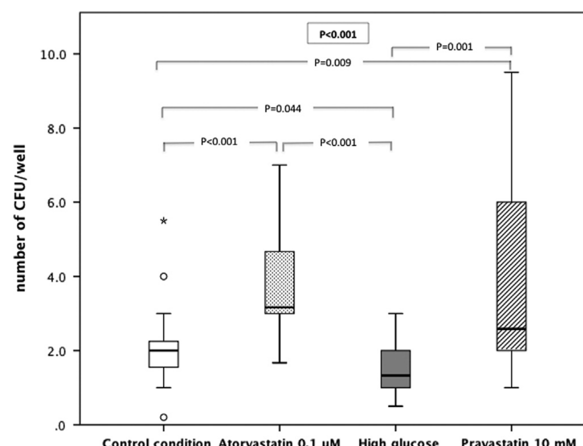

Fig. 2. Comparison of colony-forming units (CFU) between the 4 different culture conditions. Boxplot representation of CFU of early EPCs under: (1) normal glucose concentration (control condition); (2) exposure to atorvastatin 0.1 μM; (3) high glucose concentration (25 mM) and (4) supplementation with pravastatin 10 mM. Box plots represent the interquartile range of values, the horizontal lines represent 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 culture conditions. The outliers were treated as valid cases and included in the statistical analyses.

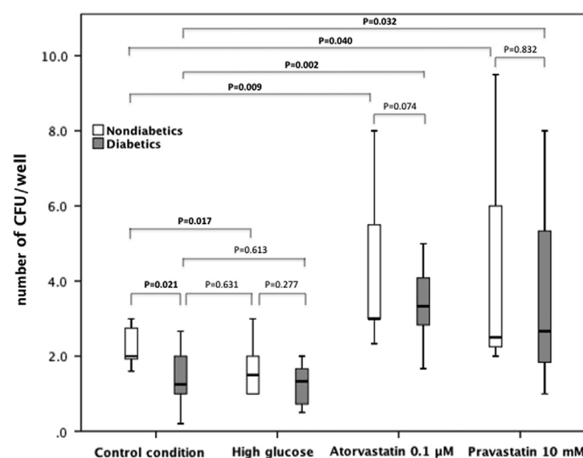

Fig. 3. Comparison of colony-forming units (CFU) capacity of cells derived from diabetics (filled bars) and non-diabetics (empty bars), grown under 4 different culture conditions. From left to right we have: (1) normal glucose concentration (control condition); (2) high glucose concentration (25 mM);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

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 ($11.7 \times 10^4 \pm 4.8 \times 10^4$ cells/ml) or with pravastatin ($12.6 \times 10^4 \pm 7.8 \times 10^4$ cells/ml) when compared to values obtained under control conditions ($7.7 \times 10^4 \pm 3.5 \times 10^4$ cells/ml) ($P=0.041$). This positive effect of statins on early EPCs survival was also observed in the diabetic subgroup (atorvastatin treatment: $11.4 \times 10^4 \pm 3.2 \times 10^4$ cells/ml; pravastatin treatment: $14.1 \times 10^4 \pm 6.8 \times 10^4$ cells/ml; control condition: $6.7 \times 10^4 \pm 2.5 \times 10^4$ 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 ($8.4 \times 10^4 \pm 3.9 \times 10^4$ cells/ml in non-diabetic patients versus $6.7 \times 10^4 \pm 2.5 \times 10^4$ cells/ml in diabetic patients, $P=0.304$).

3.4.2. *Late EPCs*

The survival curve of late EPCs showed a general downward trend, under 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. In addition, no significant differences were found between the diabetic and non-diabetic subgroups ($5.2 \times 10^4 \pm 3.4 \times 10^4$ in cells from diabetics versus $5.0 \times 10^4 \pm 1.7 \times 10^4$ 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 labeling, statin treatment induced nearly a 2-fold increase in the proportion of cells in S-phase when compared to control conditions (atorvastatin: $13.8 \pm 4.5\%$; control: $7.9 \pm 3.3\%$, $P=0.048$ and pravastatin: $15.7 \pm 1.1\%$; control: $7.9 \pm 3.3\%$, $P=0.025$) (Fig. 5A). In addition, cells from diabetic patients under control conditions showed a significantly reduced proportion of late EPCs in S-phase at day 15 (diabetics: $9.4 \pm 4.0\%$; non-diabetics: $21.9 \pm 6.0\%$, $P=0.026$) (Fig. 5B).

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. 6A). In addition, there were no significant differences between the MFI values obtained for cells derived from diabetic and non-diabetic patients, under 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. In addition, 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

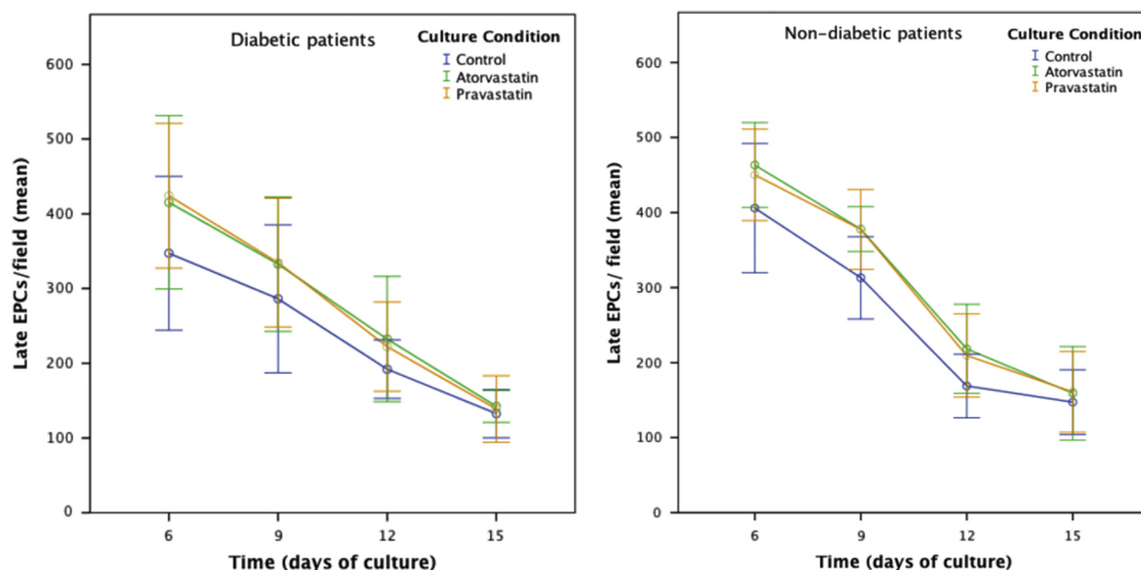

Fig. 4. Effect of statins on survival curves of late EPCs from diabetic (left chart) and non-diabetic (right chart) patients. All cultures were initially set at the same cell density (5×10^6 cells per well). Every 3 days, starting from day 6, cells from 10 randomly selected high-power fields of a phase-contrast microscope, were quantified using ImageJ 1.45 s software. Blue lines represent 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

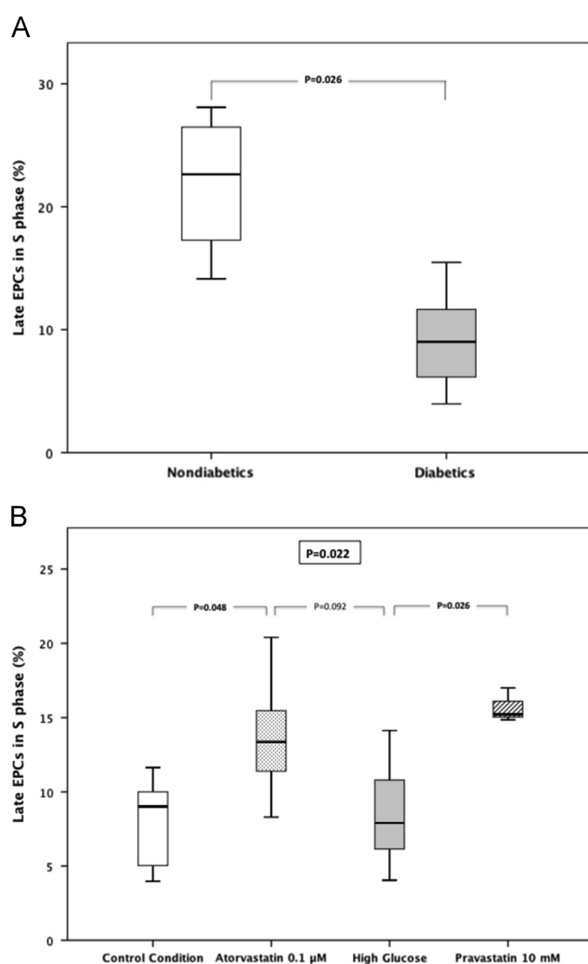

Fig. 5. Cell cycle analysis of late EPCs by DRAQ5 staining at day 15. (A) Comparison of the proportion of late EPCs on phase S between the diabetic and non-diabetic subgroup (Mann–Whitney *U* test). (B) Comparison of the proportion of late EPCs in S-phase amongst the different culture conditions. Kruskall–Wallis 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 represent the median, and whiskers represent the maximum and minimum values.

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. 6B).

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 under 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

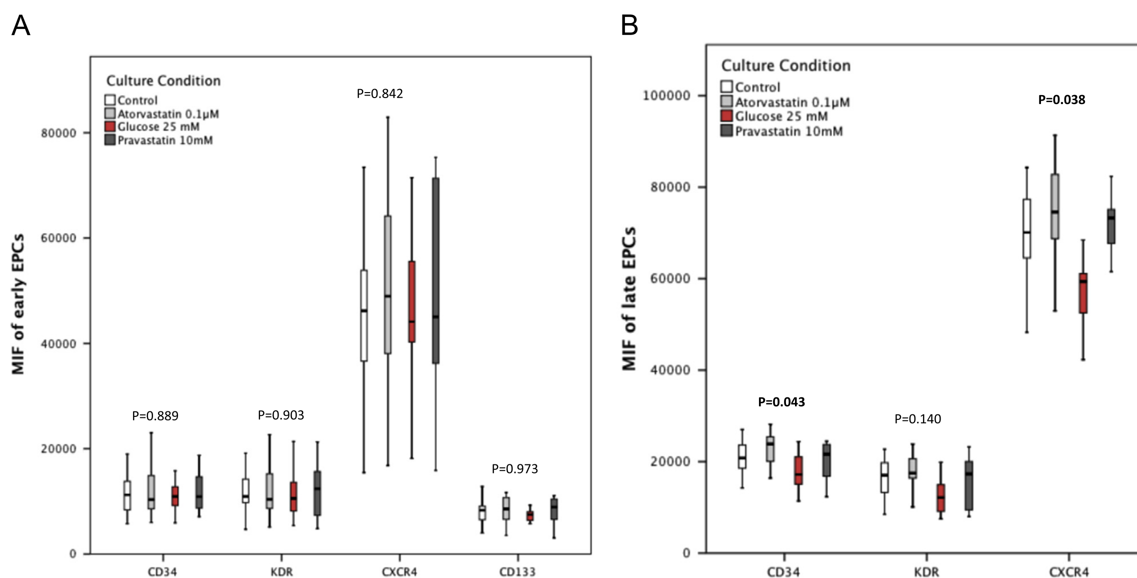

Fig. 6. Comparison of surface markers expression, assessed by mean fluorescence intensity (MIF), between EPC cultivated under 4 different culture conditions: (1) control culture condition (normal glucose concentration) - empty bars; (2) atorvastatin supplementation - light gray bars; (3) high glucose concentration exposure - red bars; (4) pravastatin supplementation - dark gray 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 represent 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 under 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 (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

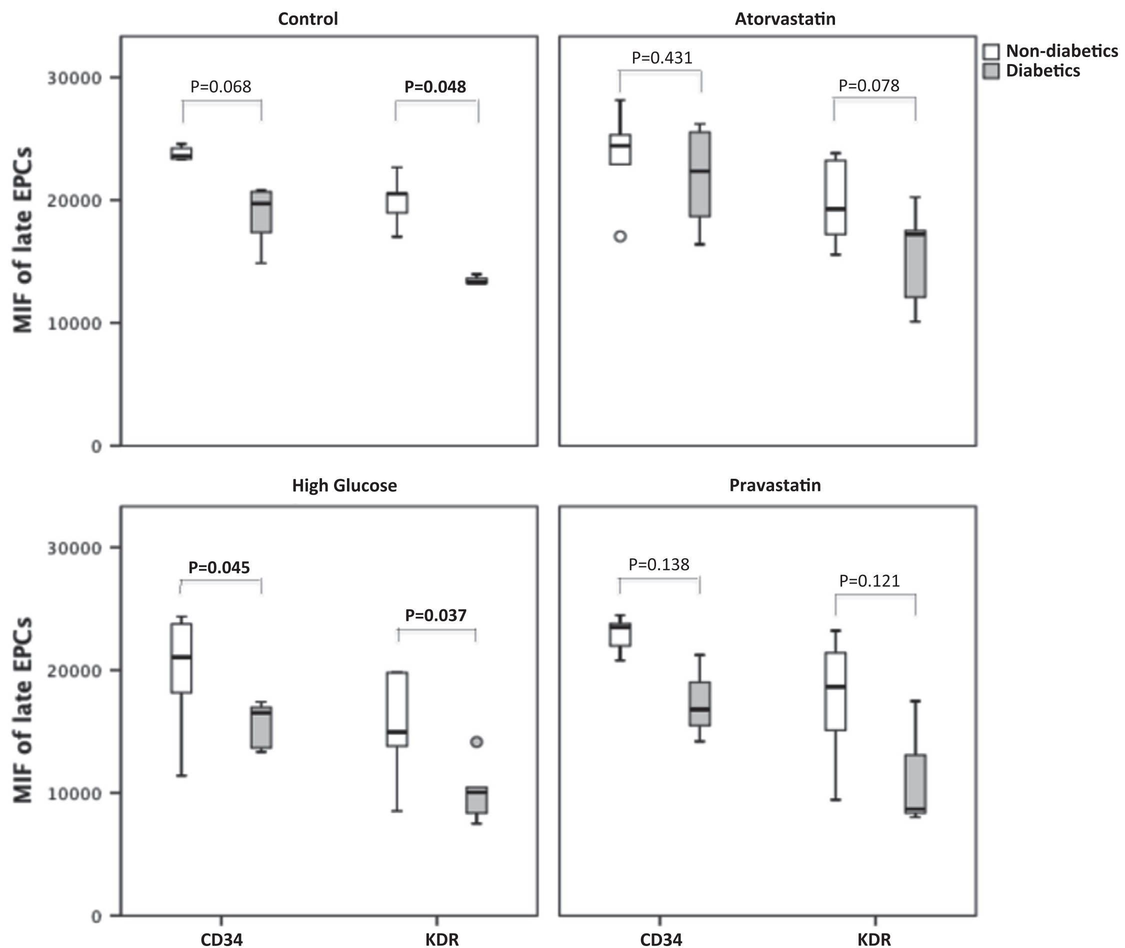

Fig. 7. Comparison of CD34 and KDR expression, assessed by mean fluorescence intensity (MIF), between late EPCs from diabetic (filled bars) and non-diabetic patients (empty bars). The comparison was performed regarding 4 different culture conditions: (1) control condition (normal glucose concentration) – 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 represent 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.

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. (2001) 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. 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. (2007) indicated that chronic statin therapy significantly reduces EPCs numbers and does not modify CFU capacity in patients with CAD . 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 under 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.

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.

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PART IV

CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the overall data of this thesis, several conclusions can be drawn:

- The *in vivo* study confirms that diabetic patients have strikingly reduced levels of circulating EPCs in the early phases of an AMI as compared to their non-diabetic counterparts. It further extends our knowledge on EPCs by demonstrating that the fraction of EPCs coexpressing the homing receptor CXCR-4 is also significantly reduced in diabetic patients, suggesting an impaired homing capacity to the sites of needed neovascularization in the ischemic tissue and damaged endothelium, in DM. Additionally, by comparing for first time, EPCs from diabetic patients with those of matched non-diabetic patients with an AMI, the *in vitro* study shows that DM has a significant negative impact on several functional parameters of EPCs, namely on their proliferation, expression of hallmark surface markers and homing capacity.

  In summary, DM dramatically impairs the response of endogenous EPCs to an AMI, by affecting both numbers and function of circulating EPCs.

- The mechanisms that regulate EPCs release and maintenance in peripheral blood, as well as the mechanisms underlying the reduction of circulating EPCs numbers and their functional impairment in diabetic patients, remain to be clearly elucidated. This thesis has helped to clarify the mechanisms involved in EPCs impairment in DM. Until now there were no data available on the relation between different populations of EPCs according to their maturation state, in diabetic patients. This relation is of obvious interest since it can help to elucidate the mechanisms underlying the impaired response of EPCs to an AMI in diabetic patients. If the mechanism responsible for the reduction in circulating EPCs levels was an impaired mobilization from bone marrow, it would be expected to have a decrease, not only in the more mature EPCs but also in the more immature ones. However, if the cause of this reduction was a reduced survival in the peripheral circulation, it would be expected to have reduced levels of the more mature EPCs but normal, or even increased, levels of the more immature populations, due to positive feedback stimulation of bone marrow recruitment. The simultaneous quantification of both circulating CD45dimCD34+KDR+ EPCs and the more immature populations of CD45dimCD34+CD133+KDR and CD45dimCD133+KDR+ progenitors shows that the reduction in the more mature EPCs population was not accompanied by the expected compensatory upregulation of the more immature ones, suggesting that the reduced response of diabetic EPCs to an AMI is due to impaired bone marrow mobilization and not simply a result of decreased survival.

  Taken together, the results of this translational research suggest as underlying mechanisms of EPCs impairment in diabetes: a reduction of EPCs mobilization from the bone marrow, a decreased proliferative capacity, and an impaired homing ability, but not a decrease in survival.

- This thesis first describes that even pre-diabetes negatively influence the response of EPCs to an AMI. The present results demonstrate that in the acute phase of a MI, circulating EPCs

numbers were significantly reduced in pre-diabetic patients and further reduced in diabetic patients as compared with patients with NGM. Nevertheless, the levels of the more immature EPCs and the proportion of EPCs coexpressing the homing marker CXCR-4 were not reduced in pre-diabetic patients, suggesting that pre-diabetes negatively impact on EPCs survival, but not on their homing processes or bone marrow recruitment.

Therefore, it is now evident that the impaired response of EPCs to an AMI is an early event in the natural history of DM, being already compromised in pre-diabetic patients. The reduction in circulating EPCs numbers is gradual, accompanying the glycemic continuum.

- Another important conclusion of this dissertation is that the degree of glycemic control is an important determinant of circulating EPCs levels in AMI patients. In the present work, numbers of all EPCs populations are negatively correlated with glycosylated hemoglobin, reinforcing the importance of the glycemic control to try to improve EPCs-mediated neovascularization in diabetic patients with an AMI.

- The finding that experimental hyperglycemic-like conditions induce dysfunction in EPCs from non-diabetic AMI patients *in vitro*, combined with the observation of an inverse correlation between glycemic levels and circulating EPCs levels *in vivo*, support the hypothesis that chronic hyperglycemia per se is sufficient to jeopardize the endogenous response of EPCs to an AMI.

- The present research indicates that chronic insulin therapy might improve EPCs response to myocardial ischemia in diabetes, probably through a mechanism beyond glycemic control. In fact, despite the longer DM duration and the worse glycemic control, insulin-treated diabetic patients tend to present similar circulating levels of the more mature CD45dimCD34+KDR+ EPCs to those of non-diabetic patients in the acute phase of an MI, suggesting that chronic insulin therapy attenuates the expected deficit in circulating EPCs of diabetic patients. Since levels of the more immature EPCs populations and the fraction of EPCs coexpressing the homing marker CXCR-4 appear to be unchanged by insulin therapy, an increase in survival of the more mature population of EPCs seems to be the most likely mechanism involved in the potential benefit of insulin therapy over EPCs *in vivo*. However, it is still unknown whether, under better glycemic control, chronic insulin therapy could completely reverse EPCs dysfunction of diabetic patients and normalize their response to an AMI.

- Through the combination of both *in vitro* and *in vivo* studies, this thesis provides evidence that statin therapy remarkably increases the levels of circulating EPCs and significantly improves their function, in the clinical setting of an AMI. Moreover, it indicates that this stimulation of EPCs by statins may be, at least in part, mediated through their well-known anti-inflammatory action. The *in vitro* exposure of EPCs to statins consistently improve several functional parameters of EPCs, such as proliferation, survival and expression of the homing marker, CXCR-4. It is worth noting that in our cohort of AMI patients, prior chronic statin therapy enhanced EPCs response to myocardial ischemia, even in diabetic patients. Furthermore,

despite the profound dysfunction of EPCs associated with diabetes, the *in vitro* functional benefits of statins over EPCs were also verified in cells from diabetic patients.
In summary, this thesis indicates that statin therapy can correct the functional impairment of EPCs from diabetic patients, excluding therefore, the hypothesis of an irreversible "hyperglycemic memory" effect of diabetes over EPCs.

- Finally, this work suggests that statin stimulation of the endogenous EPCs response to an AMI is dose-dependent. In this research, we compared for the first time the effect of different intensities of statin therapy at discharge on the evolution of circulating EPCs levels after an AMI, verifying that only the high-intensity statin regimen prevents the expected decrease of circulating EPCs levels during the post-MI period. Therefore, it is tempting to speculate that high-intensity statin therapy can counteract the expected decline in EPCs levels following an AMI, because it is a so strong stimulus for EPCs recruitment, that it parallels the stimulation mediated by myocardial ischemia in the acute phase of a MI. In a clinical point of view, these findings strongly reinforce the importance to follow the current recommendation, and start high-intensity statin therapy early in all patients with AMI.

Taken together the results presented in this thesis may have important clinical implications, since they demonstrate that the profound impairment of EPCs associated with DM can be pharmacologically reverted. Therefore, pharmacological strategies to enhance the endogenous response of EPCs in diabetic patients with an AMI appear as a realistic goal and can be actively pursued in an attempt to improve the worse outcomes of this increasing population.

This dissertation provides many answers but raises even more questions. In order to elucidate the molecular mechanisms underlying the regenerative role of EPCs *in vivo* and to identify the specific targets for appropriate patient-tailored pharmacological modulation, there are still open issues that need to be addressed in future research. Do EPCs directly participate in regeneration of the damaged endothelium or simply act indirectly through paracrine mechanisms? Endothelial dysfunction is known to be associated with increased vascular inflammation. Is inflammation a causative player or just a marker of EPCs impairment in DM? It has been recently suggested that non-coding RNAs or microRNA (miRNAs) are key players in the pathogenesis of hyperglycemia-induced vascular damage and that the deregulation of specific miRNAs expression may contribute to vascular disease in DM. Can the pharmacological modulation of specific miRNAs improve EPCs-driven repair in diabetic patients? Which are the mechanisms of action, at a molecular level, needed to completely normalize the endogenous EPCs pool by pharmacological therapy and therefore, ameliorate the native response of EPCs to an AMI in diabetic patients?

Looking towards the future, further investigation on other promising drugs, including new oral antidiabetics and antianginal agents, might provide valuable insights for unlocking our innate regenerative potential, contributing for the progress of the promising field of regenerative pharmacology.