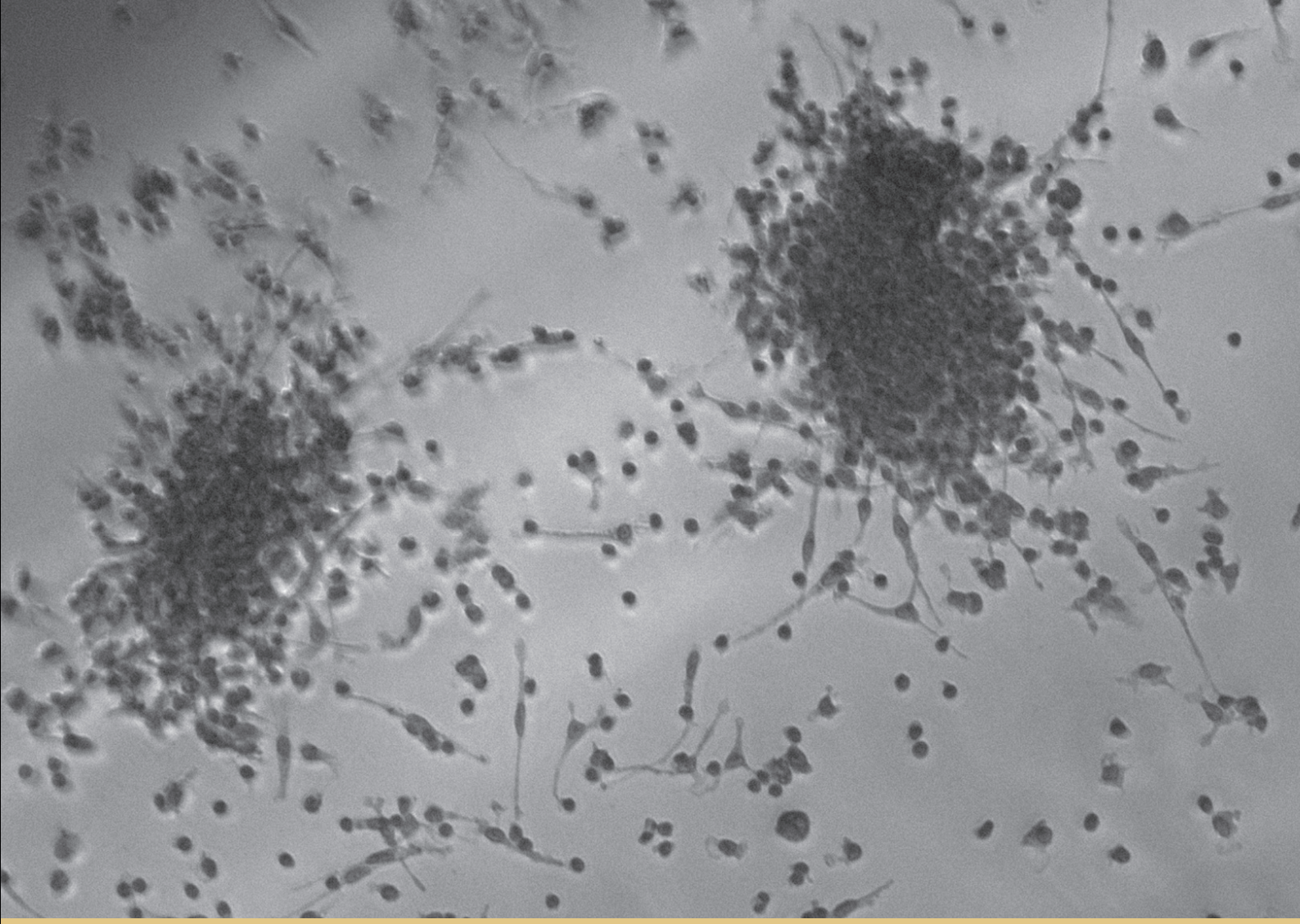

Natália Sofia Cláudio António

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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Universidade de Coimbra

NATÁLIA SOFIA CLÁUDIO ANTÓNIO

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

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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, especialidade de Ciências Fisiológicas (Farmacologia) realizada sob a orientação científica do Prof. Doutor Carlos Fontes Ribeiro, 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.

II. **António N**, Fernandes R, Ribeiro CF, Providência LA. Challenges in vascular repair by endothelial progenitor cells in diabetic patients. Cardiovasc Hematol Disord Drug Targets. 2010;10(3):161-6.

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.

IV. **António N**, Fernandes R, Soares A, Soares F, Lopes A, Carvalheiro T, Paiva A, Mariano Pêgo G, Providência LA, Gonçalves L, Fontes Ribeiro C. 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. Eur J Clin Pharmacol. 2014;70(10):1181-93.

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 postnatal 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
DiI	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

Eur J Clin Pharmacol. 2010; 66(3):219-30

CHAPTER II

Challenges in vascular repair by endothelial progenitor cells in diabetic patients

Cardiovasc Hematol Disord Drug Targets. 2010;10(3):161-6

CHAPTER III

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

Cardiovasc Diabetol. 2014;13:101

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CARDIO VASCULAR DIABETOLOGY

ORIGINAL INVESTIGATION Open Access

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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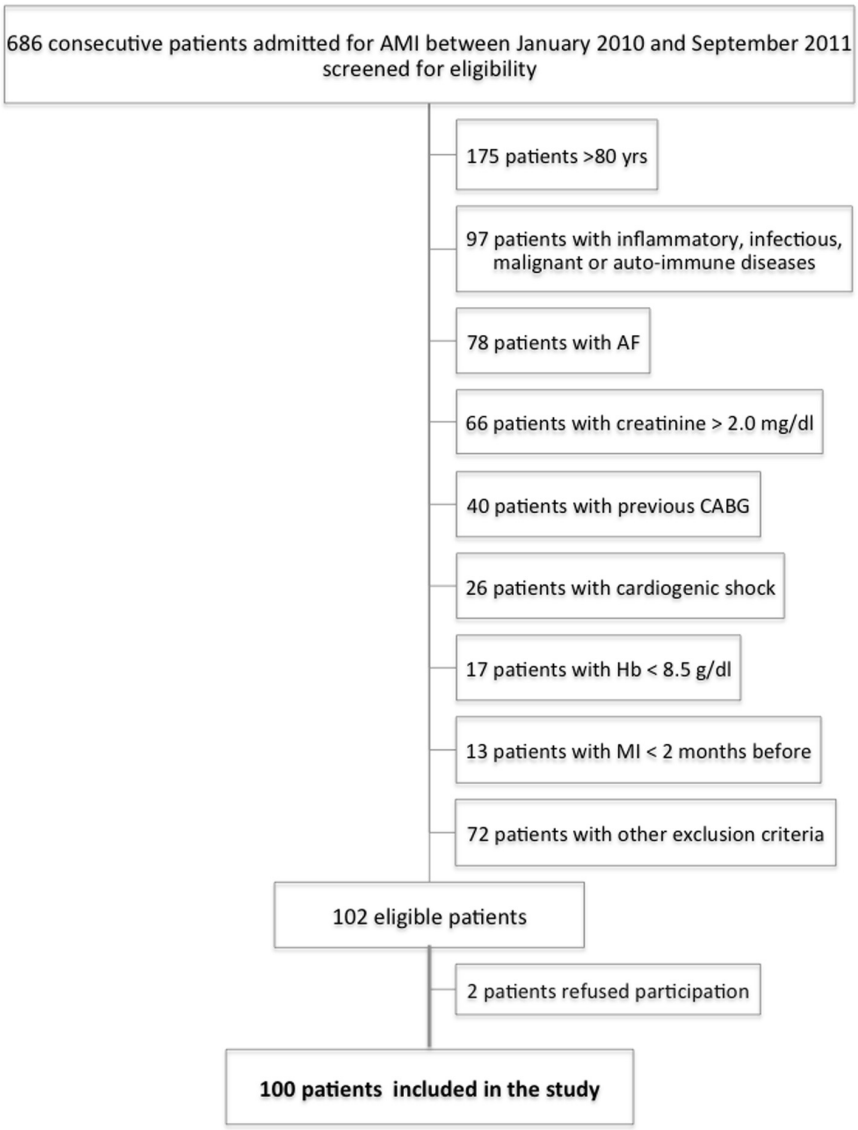

Figure 1 Flow diagram of patient recruitment. AF, atrial fibrillation; AMI, acute myocardial infarction; CABG, coronary artery bypass graft; Hb, hemoglobin; MI, myocardial infarction; yrs, years old.

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, pre-diabetes 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

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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 non-diabetic 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.

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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+CD133+KDR+ EPCs to around half the levels of nondiabetics, and numbers of its precursors CD45dimCD133+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 prediabetes (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 prediabetes 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 CD45dimCD133+KDR+CXCR4+) were not significantly reduced in prediabetic 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.

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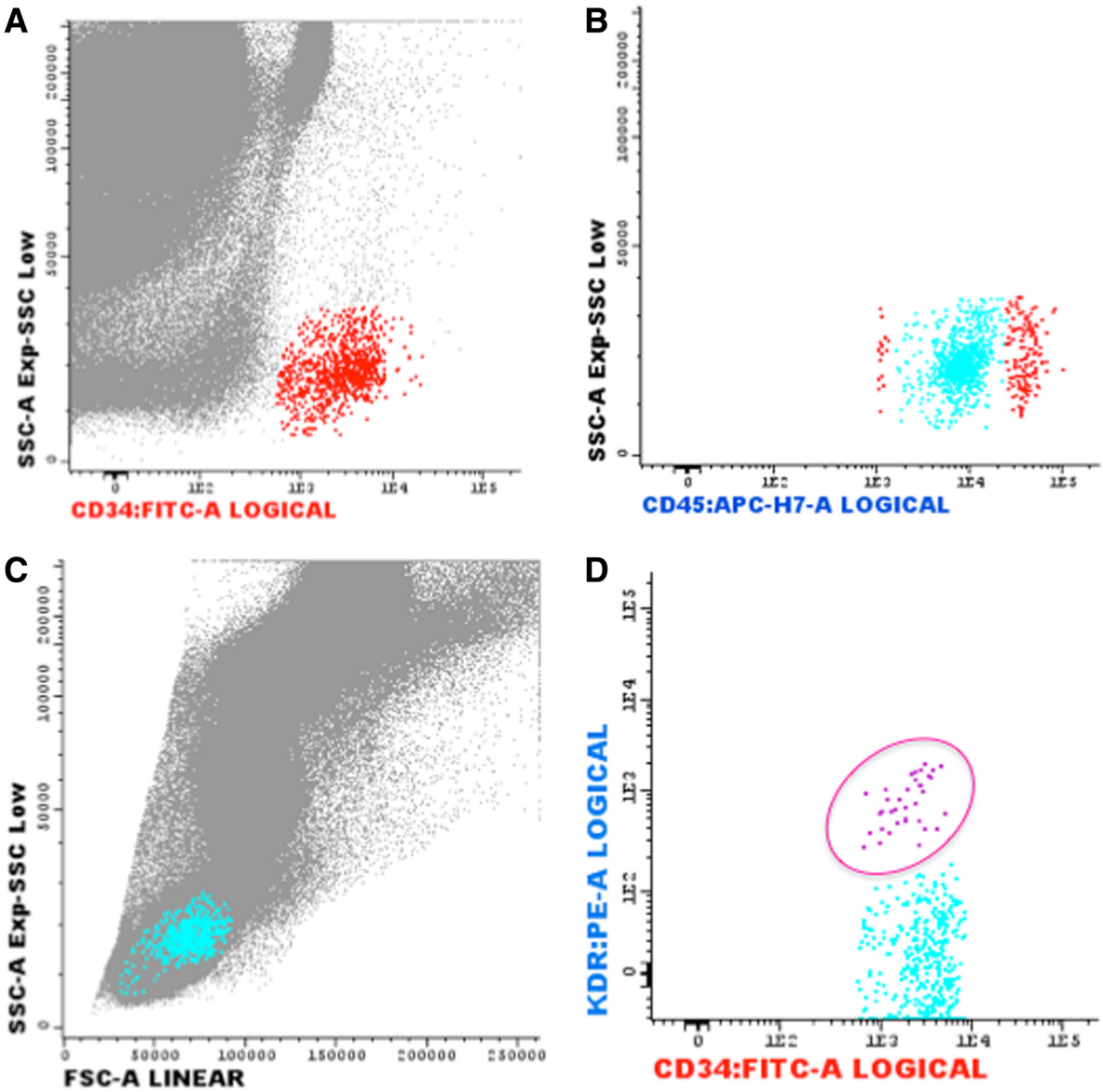

Figure 2 Representative flow cytometry quantification of CD45low/CD34+/KDR+ EPCs in a a nondiabetic patient with STEMI. (A) The cluster of CD34+ cells (red points) was analyzed using FITC-labeled antibodies against CD34 vs. SSC. **B)** CD45dim subset analysis was conducted using APC-H7-labeled antibodies 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 $p = 0.004$,

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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 CD45dimCD133+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 CD45dimCD34+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

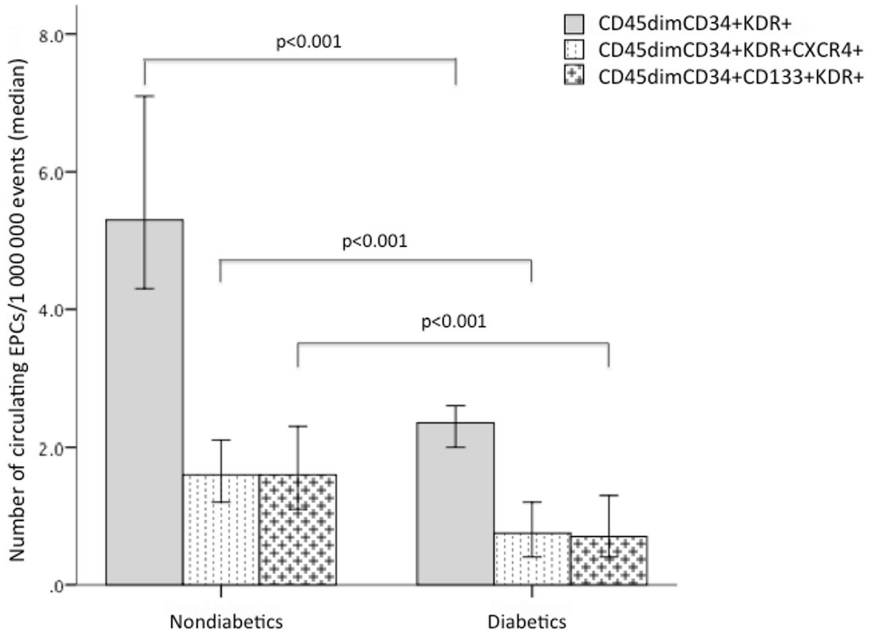

Figure 3 Comparison of circulating EPCs levels between diabetics and nondiabetics. Bars represent median and error bars interquartile range of circulating EPCs numbers quantified by flow cytometry. Mann Whitney U test was used for the comparison between diabetic and nondiabetic EPCs levels.

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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,

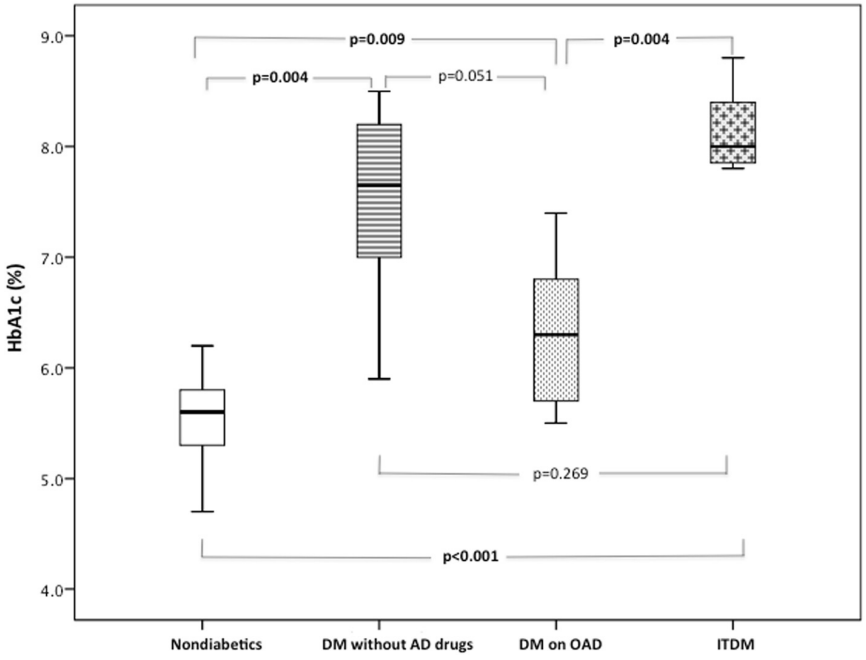

Figure 4 Glycosylated hemoglobin levels according to chronic antidiabetic treatment. Box plots represent the interquartile range of values, the horizontal lines show the median, and whiskers represent the maximum and minimum values. In order to find between which antiadiabetic treatment categories there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

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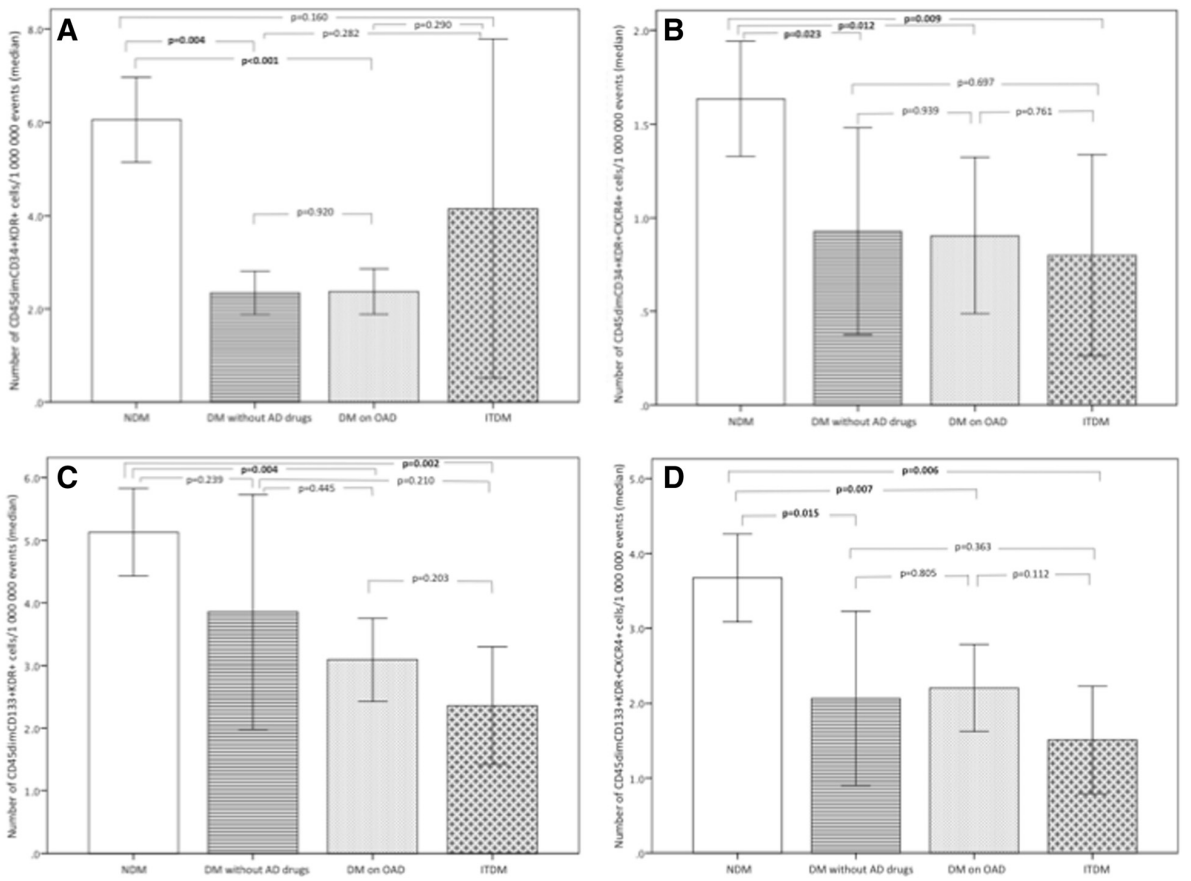

Figure 5 Comparison of circulating EPCs levels between nondiabetic patients and diabetics under different antidiabetic treatments. **(A)** Circulating numbers of CD45dimCD34+KDR+ cells; **(B)** Circulating numbers of CD45dimCD34+KDR+CXCR4+ cells; **(C)** Circulating numbers of CD45dimCD133+KDR+ cells 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

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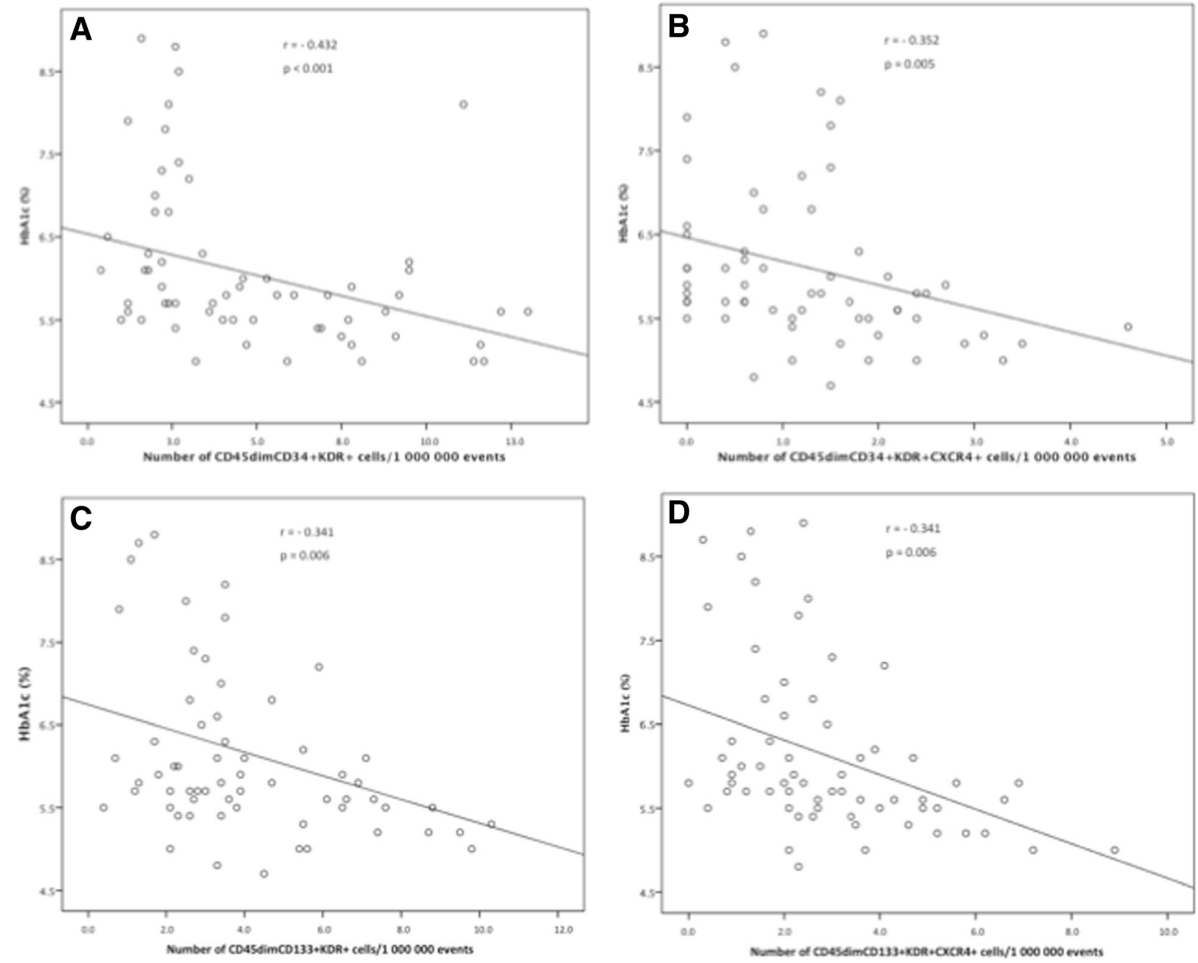

Figure 6 Relationship between levels of circulating EPCs and HbA1c. Scatter plots showing significant negative relationship relationship (Pearson correlation) between HbA1c and circulating levels of: **A)** CD45dimCD34+KDR+ cells; **B)** CD45dimCD34+KDR+CXCR4+ cells; **C)** CD133+KDR+ cells; **D)** CD133+KDR+CXCR4+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

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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.

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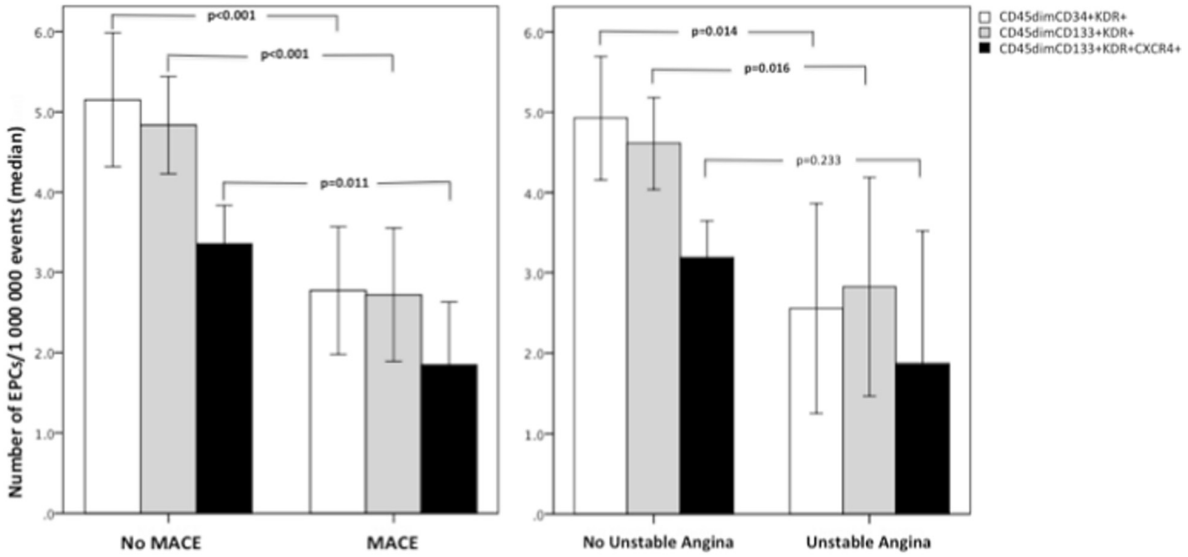

Figure 7 Comparison of baseline EPCs levels between patients with or without cardiovascular events during the 2-year follow-up period. Bars represent median and error bars interquartile range of circulating EPCs numbers, quantified by flow cytometry within the first 24 h of admission. We compared EPCs 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+CD 133+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+CX CR4+ 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

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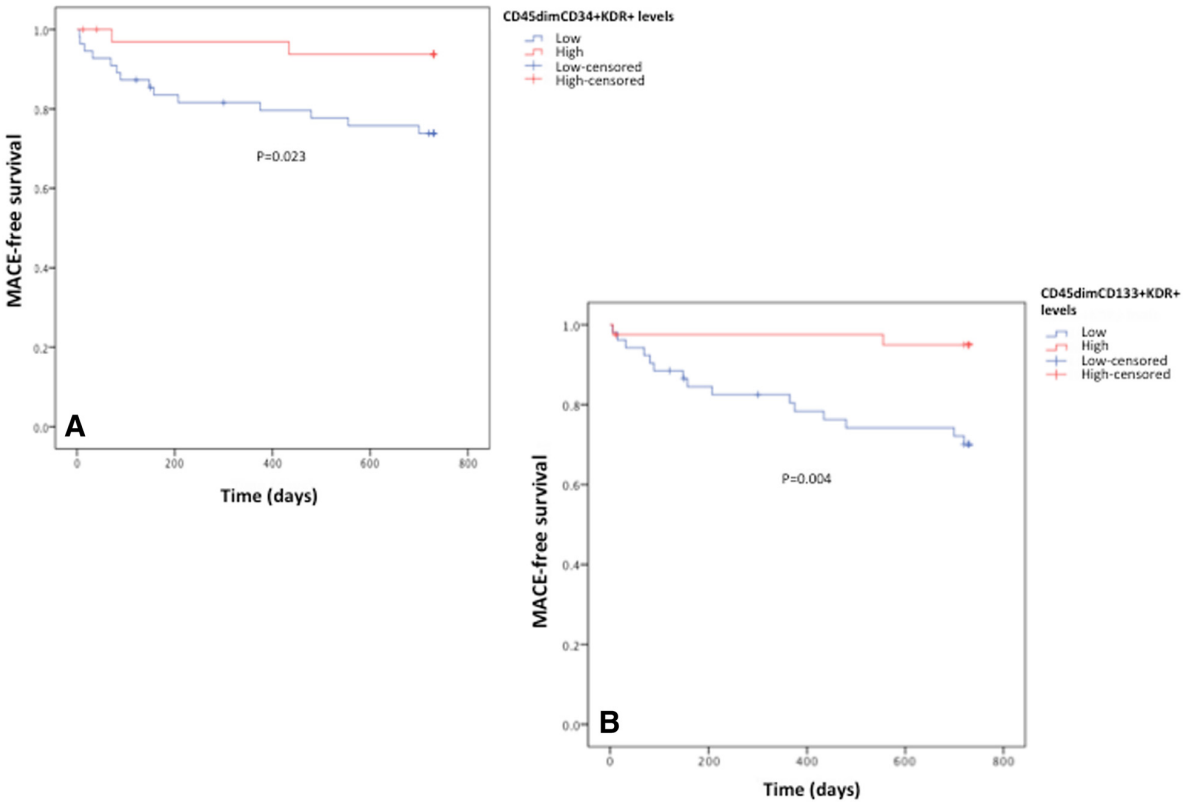

Figure 8 Kaplan-Meier MACE-free survival curves following an AMI, according to circulating EPCs levels presented in the acute phase. **A)** Comparison of MACE-free survival curves between patients with levels of CD45dimCD34+KDR+ cells above (red line) or below (blue line) the median. **B)** Comparison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

Eur J Clin Pharmacol. 2014; in press

CHAPTER V

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

Eur J Pharmacol. 2014; in press

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.