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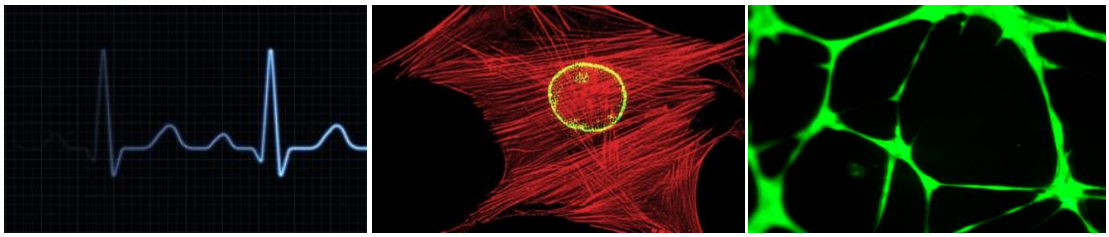

Comprehensive Characterization of Human Mesenchymal Stem/Stromal Cells From Healthy Donors and Acute Myocardial Infarction Patients

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Abstract

Cardiovascular Diseases (CVDs), namely Acute Myocardial Infarction (AMI), are the leading cause of death worldwide. Current treatments of AMI are incapable of regenerating the necrotic tissue resultant from ischemia. Regarding this issue, Mesenchymal Stem/Stromal Cells (MSCs) have shown some promising results in early clinical trials. Autologous MSCs may not represent the best option for a MSC-based therapy since their performance can be impaired by the associated comorbidities, allied to the age, of AMI patients. The use of Bone Marrow (BM)-derived cells can also be potentially limiting since it involves an invasive procedure for cell collection; Adipose Tissue (AT) may represent an alternative promising source of MSCs for AMI settings.

The aim of this work was to establish a comparative study between BM-derived MSCs from AMI patients, and BM-, AT-derived MSCs from healthy donors in terms of proliferative, angiogenic, secretory, and oxidative stress resistance potential, in order to identify the most adequate cell type for the clinical setting. Additionally, two different methods for the isolation of Adipose-derived Stem/Stromal Cells (ADSCs) were studied.

Healthy BM MSCs presented a higher secretory and angiogenic potential, while AMI BM MSCs had the highest proliferative capacity. The incubation of MSCs under hypoxia (2% O_2) boosted the aforementioned results. Further studies should be conducted to investigate the mechanisms underlying the improved *in vitro* proliferative capacity of AMI BM MSCs demonstrated herein. Concerning ADSCs isolation, these cells might be isolated from AT through a non-enzymatic method, albeit the slight lower yield when compared to the standard enzymatic-based protocol.

Keywords: Acute Myocardial Infarction, Mesenchymal Stem/Stromal Cells, Bone Marrow, Adipose Tissue, Angiogenesis, Hypoxia

Resumo

As doenças Cardiovasculares (CVDs), como o Enfarte Agudo do Miocárdio (AMI), são a principal causa de morte mundialmente. Os tratamentos atuais do AMI são incapazes de regenerar o tecido necrótico resultante da isquemia. As Células Estaminais/Estromais Mesenquimais (MSCs) têm mostrado resultados promissores em ensaios clínicos piloto. O desempenho de MSCs autólogas pode ser afectado por comorbidades associadas, aliadas à idade, dos doentes de AMI. O uso de células derivadas da Medula Óssea (BM) pode ser ainda potencialmente limitante dado envolver uma colheita invasiva; o Tecido Adiposo (AT) pode representar uma fonte alternativa de MSCs no contexto do AMI.

O objetivo deste trabalho foi estabelecer a comparação entre MSCs derivadas da BM de doentes AMI, e MSCs derivadas de BM e AT de dadores saudáveis relativamente ao potencial proliferativo, angiogénico, secretório, e de resistência ao stress oxidativo, para identificar o tipo de célula potencialmente mais adequado ao cenário clínico. Adicionalmente, estudaram-se dois métodos diferentes de isolamento de Células Estaminais/Estromais-derivadas de Tecido Adiposo (ADSCs).

As BM MSCs saudáveis demonstraram o potencial secretório e angiogénico mais elevado, enquanto as AMI BM MSCs mostraram uma capacidade de proliferação superior. A incubação das MSCs em hipóxia (2% O_2) melhorou os resultados mencionados. Serão necessários estudos adicionais para investigar os mecanismos pelos quais as AMI BM MSCs demonstraram uma capacidade proliferativa *in vitro* superior nas condições deste estudo. Relativamente ao isolamento de ADSCs, estas podem ser isoladas de AT por um método não enzimático, apesar do rendimento ligeiramente inferior ao protocolo padrão baseado num método enzimático.

Palavras-chave: Enfarte Agudo do Miocárdio, Células Estaminais/Estromais Mesenquimais, Medula Óssea, Tecido Adiposo, Angiogénese, Hipóxia

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List of Abbreviations

AA	Antibiotic-Antimycotic
ACE	Angiotensin-Converting Enzyme
ADP	Adenosine Diphosphate
ADSCs	Adipose-derived Stem/Stromal Cells
AMI	Acute Myocardial Infarction
ASCs	Adult Stem Cells
AT	Adipose Tissue
ATP	Adenosine Triphosphate
Bcl-2	B-cell lymphoma-2
bFGF	basic Fibroblast Growth Factor
BM	Bone Marrow
BM-MNCs	Bone Marrow Mononucleated Cells
BM-MSCs	Bone Marrow Mesenchymal Stem/Stromal Cells
CABG	Coronary Artery Bypass Grafting
CD	Cluster of Differentiation
CFI	Cumulative Fold Increase
CFU-F	Colony-Forming Unit-Fibroblast
CFUs	Colony-Forming Units
CHD	Coronary Heart Disease
CK	Creatine Kinase
CKMB	Creatine Kinase MB isoform
CMCs	Cardiomyocytes
CPCs	Circulating blood–derived Progenitor Cells
CPD	Cumulative Population Doublings
CRP	C-Reactive Protein
cTn	Cardiac Troponin
CVDs	Cardiovascular Diseases
DiI-Ac-LDL	DiI-Acetylated-Low-Density Lipoprotein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECG	Electrocardiogram
ECM	Extracellular Matrix
ECs	Endothelial Cells
EF	Ejection Fraction
EGF	Epidermal Growth Factor
EGM	Endothelial Growth Medium
ELISA	Enzyme-Linked Immunosorbent Assay

EPCs	Endothelial Progenitor Cells
EPDCs	Epicardially Derived Cells
ESCs	Embryonic Stem Cells
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCT	Fundação para a Ciência e Tecnologia
FI	Fold Increase
FITC	Fluorescein Isothiocyanate
G-CSF	Granulocyte Colony-Stimulating Factor
GVHD	Graft-Versus-Host-Disease
HBSS	Hank's Buffered Salt Solution
HGF	Hepatocyte Growth Factor
HSCs	Hematopoietic Stem Cells
HSP27	Heat Shock Protein 27
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
ICMP	Ischemic Cardiomyopathy
IFN-γ	Interferon Gamma
IGF-1	Insulin-like Growth Factor 1
IHD	Ischemic Heart Disease
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IMDM	Iscove's Modified Dulbecco's Medium
INT	2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phe-nyltetrazolium chloride
iPSCs	induced Pluripotent Stem Cells
ISCT	International Society for Cellular Therapy
isl-1	islet-1
KIR	Killer cell Immunoglobulin-like Receptors
LDH	Lactate Dehydrogenase
LIF	Leukemia Inhibitory Factor
Lin^-	Lineage-negative
MACS	Magnetic Activated Cell Sorting
MDR1	Multidrug Resistance Protein 1
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
MSCs	Mesenchymal Stem/Stromal Cells
NK	Natural Killer

NK-κB	Nuclear Factor Kappa B
NOD	Non-Obese Diabetic
NSTEMI	Non-ST Elevation Myocardial Infarction
P(0-9)	Passage (0-9)
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PBSCs	Peripheral Blood Stem Cells
PCI	Percutaneous Coronary Intervention
PD	Population Doublings
PDGF-AB	Platelet-Derived Growth Factor-AB
PE	Phycoerythrin
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PI	Propidium Iodide
PSCs	Pluripotent Stem Cells
PTCA	Percutaneous Transluminal Coronary Angioplasty
ROS	Reactive Oxygen Species
rPA	reteplase Plasminogen Activator
rpm	rotations per minute
SCA-1	Stem Cell Antigen-1
SCBL-RM	Stem Cell Bioengineering and Regenerative Medicine Laboratory
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficient
SDF-1	Stromal Cell-Derived Factor 1
SEM	Standard Error of the Mean
SKMs	Skeletal Myoblasts
SP	Side Population
STEMI	ST Elevation Myocardial Infarction
SVF	Stromal Vascular Fraction
TEI	Transendocardial Injection
TGF-β	Transforming Growth Factor Beta
TLR	Toll-Like Receptors
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor Alpha
TNK-tPA	Tenecteplase tissue Plasminogen Activator
tPA	tissue Plasminogen Activator
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
TXA_2	Thromboxane A_2
URL	Upper Reference Limit
VEGF	Vascular Endothelial Growth Factor

XSF	Xeno(geneic), Serum-Free
α-MEM	MEM Alpha Medium

I. Introduction

I.1 Motivation

Our society has evolved century after century, and experienced crucial revolutions, especially along the last ones[1]. The industrial revolution of the 18th century, which happened in the currently nominated developed countries, had significant impacts in the societies of that time, namely at an economic level[2]. The improvement of socioeconomic, sanitation and nutrition conditions conducted to a shift in the infirmities that affected the populations of such countries[2]. This shift, coined by Abdel Omran as 'epidemiologic transition', became evident during the 19th century, with a reduction of infectious diseases and maternal and nutritional disorders, accompanied by an increase of non-communicable diseases[2]. Such transition is ongoing in developing countries, although motivated essentially by the importation of medical technology[2].

Amongst the non-communicable diseases, the new plague of the 21st century, Ischemic Heart Disease (IHD), a disorder belonging to the group of Cardiovascular Diseases (CVDs)[3], appears as the main cause of death worldwide[4]. In 2001, 11.8% and 17.3% of all deaths in low/middle and high income countries, respectively, were caused by this disease[4]. During the year of 2009, IHD was the second most fatal disease in Portugal, after CVD, responsible for 7 558 deaths in a total of 104 964 registered deaths[5]. Although these numbers are already impressive, those are expected to increase in the future[6]. The worldwide decline of fertility, from 5.0 children per women in 1950 to 1955 to the estimate of 2.2 or 1.8 children per women in 2045 to 2050, is a great contributor to the aging of population[7]. The reduction of child mortality and the increase in life expectancy, which is expected to change from 65 years in 1950 to 83 years in 2045 to 2050 in developed countries, also have an additive effect to this phenomenon[7]. The final result is a projection of 32%, 19% and 10% of people 60 or older in 2050, in developed, less developed and least developed regions, respectively, against 23%, 9% and 5% in 2013[7]. Knowing that cardiovascular and circulatory diseases have a higher incidence in a population over 50 years old (Figure I-1)[8], and despite the expected decline in age-specific death rates for this disease, the population aging will then be a major factor for the increase of cardiovascular deaths from 16.7 million in 2002 to 23.0 million deaths worldwide in 2030[6].

Beyond the high mortality associated, CVDs also induce a significant disability to the survivor patients[4], thus representing a substantial economic burden, both directly and indirectly[9]. Considering medical expenditures as direct costs and foregone earnings resultant from work loss among currently employed individuals or individuals incapable of working, home productivity loss and premature mortality as indirect costs, Heidenreich and colleagues (2011) projected the evolution of the economic burden for CVDs in the United States[9]. For the year of 2030, direct and indirect costs were estimated to increase respectively 200% and 61%, relatively to those from 2010, exceeding a total of 1$ trillion[9]. These numbers and previously presented statistics highlight the importance of not only avoiding risk factors to prevent the disease[9; 10] but also of improving the current treatments[11] in a joint action between clinicians and researchers, in order to reduce the economic burden of CVDs, by minimizing their mortality and maximizing the quality of life of patients.

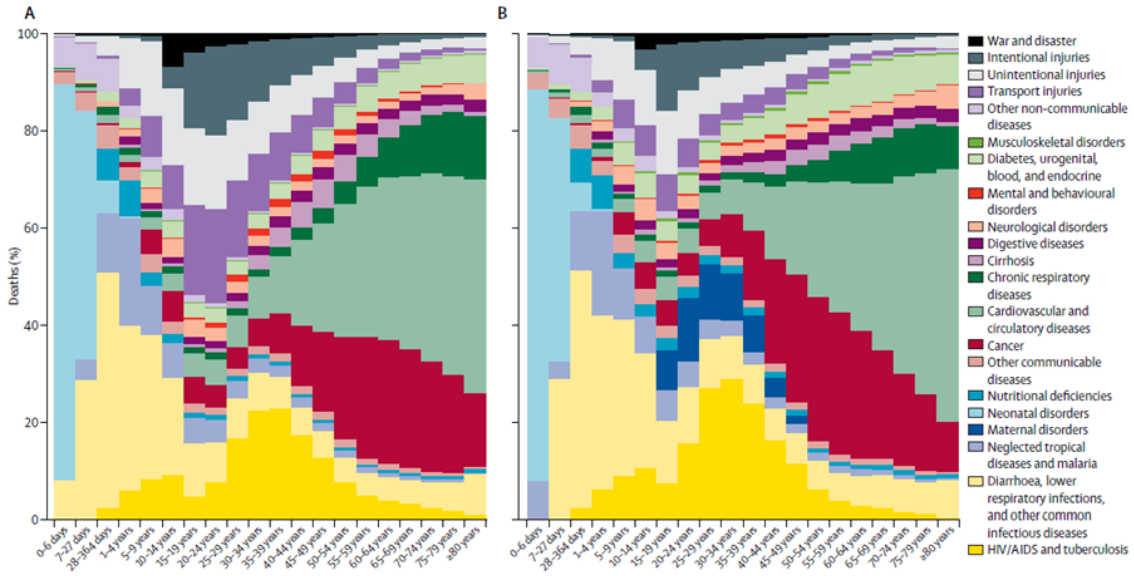


Figure I-1. Percentage of global deaths for female and male individuals in 1990 and 2010 by cause and age. (A) Male individuals, 2010. (B) Female individuals, 2010 (adapted)[8].

I.2 Cardiovascular Diseases

I.2.1 Definition and Classification

The circulatory system comprises the heart and the blood vessels, and its purpose is the delivery of oxygen and nutrients throughout the body and the removal of metabolic waste products in exchange[12]. The heart, which is composed of a muscle called myocardium, works uninterruptedly to pump blood to the body[12]. CVD is the name given to a group of several diseases that affect heart and/or blood vessels[3]. The most relevant diseases for this work will be described above.

Atherosclerosis, or **Arteriosclerotic Heart Disease**, is a disorder characterized by the deposition of fat, cholesterol and cellular wastes in the interior wall of arteries of large or medium calibre[12]. These deposits, called atheroma, may be hardened through the deposition of calcium and, as they grow, they occlude the lumen of the vessels, reducing the blood flow[3]. Atherosclerosis is the basis of other CVDs such as Aortic Aneurism[3], Coronary Heart Disease (CHD), Myocardial Infarction (MI) and Peripheral Artery Disease[3; 12].

CHD, also known as IHD, rises from an atheroma in coronary arteries, which reduces their lumen, or a blockage of such vessels by blood clots resultant from the rupture of an atheroma in other larger arteries[13]. The reduction of blood flow through these arteries may lead to an insufficient supply of oxygen, called ischemia, which, in turn, may give rise to a MI[13].

MI, commonly called Heart Attack, is the result of an acute obstruction of a coronary artery[13]. This obstruction, whose origin is an atheroma, a clot or a spasm of the coronary artery[12], creates a severe ischemia that causes the necrosis of the affected myocardium and, subsequently, arrhythmias and sudden cardiac death[13].

Hypertensive Heart Disease arises from a condition called hypertension[13]. Hypertension is the high pressure in the circulatory system, defined as a systolic blood pressure higher than 140 mm Hg

and/or a diastolic blood pressure higher than 90 mm Hg[13]. In a situation of hypertension, there is greater resistance to blood flow, which increases the effort of the heart to pump the blood[13].

I.2.2 Acute Myocardial Infarction

Given the purpose of the present work – to define the *pros* and *cons* of an autologous Mesenchymal Stem/Stromal Cells (MSCs)-based therapy for Acute Myocardial Infarction (AMI) as well as to evaluate the potential of using different MSC sources and expansion conditions in this setting – it is important to describe the disease in more detail, referring the main risk factors associated with it, as well as the available treatments.

I.2.2.1 Pathophisiology

A MI is defined as the death of the cardiac muscle cells, called Cardiomyocytes (CMCs), following a prolonged ischemia[14]. Many of the MI situations arise from the development of atherosclerosis in the coronary arteries, with the atheroma plaque growing and successively reducing the lumen of those vessels[14]. Besides the existence of an atheroma plaque in the coronary arteries, a MI can also be caused by a coronary spasm, an embolus or even the dissection of those vessels, although these events are less frequent[15].

I.2.2.1.1 Rupture of the Atheroma Plaque

There are some processes that might act on the plaque and make it more prone to rupture, which usually occur at the plaque cap with a lipid pool near its lateral margin[16]. One of the mechanisms is started by the release of oxygen radicals and neutral proteases by macrophage-derived foam cells[16]. These radicals and proteases then lead to the enzymatic degradation of the connective tissue matrix that constitutes the interior of the plaque[16]. The rupture initiates a thrombotic process, with the platelets being stimulated to aggregate at the sites of endothelial dysfunction due to the increase of catecholamines, which are associated with stressful situations, including exercise and sudden fright[16]. Other agents play an important role in this thrombotic process, such as Thromboxane A_2 (TXA_2), serotonin, and Adenosine Diphosphate (ADP)[16].

I.2.2.1.2 Ischemic Environment

The thrombus that forms during the thrombotic process may partially or completely occlude the coronary artery, suspending the blood flow and, consequently, the supply of nutrients and oxygen to the surrounding myocardium[14]. In this poor environment, the production process of Adenosine Triphosphate (ATP), which is the main energy source in the cardiac tissue, changes from the normal one, through oxidative phosphorylation in the mitochondria, to the breakdown of high-energy phosphates into purines[17]. During this period, adenosine might leave the CMCs to go to the extracellular space, inducing vasodilatation, thus decreasing the oxygen demand by the heart, and increasing the glycolytic flux to produce energy[17]. With the activation of the anaerobic glycolysis, the pyruvate produced in this process turns into lactate, which creates osmotic cell stress and water accumulation, inducing sarcolemmal disruption[17]. The accumulated water will decrease the myocardial

function and activate the rennin-angiotensin-aldosterone system, which in turn will later on trigger the myocardial fibrosis[17].

The ischemic environment also contributes to the activation of the lysosomes present inside the CMCs[17]. The lysosomes then start to hydrolyse the cell membrane, allowing the deregulated entrance of positive ions, such as calcium, which will also cause sarcolemmal disruption through the same mechanisms as lactate[17]. The reduction of the pH, resulting from the inside accumulation of lactate, will also activate the Na^+/H^+ exchangers and conduct to the excessive intake of sodium that cannot be regulated by the Na^+/K^+ ATPase[17]. These processes might result in phospholipases and proteases activation and damage of membrane phospholipids and ion channels, conducting to an acceleration of the cellular necrosis[17].

When exceeding 30 minutes, the hypoxic environment may lead to an irreversible damage of the heart muscle, and the MI occurs, with the damaged area increasing as time passes by[14]. The myocardium can turn necrotic if subjected to 6 continuous hours without blood flow, and this loss of functional muscle affects the correct pumping of blood by the heart and may lead to death[14].

I.2.2.1.3 Inflammation

The inflammatory response to the AMI might be initiated by the activation of Toll-Like Receptors (TLR), the transcription Nuclear Factor Kappa B (NK-κB) and the complement system, by products of injured tissue, such as Reactive Oxygen Species (ROS) and proteins released from necrotic cells[18]. TLR are pattern recognition receptors that have their signalling pathway activated in situations of heart failure, especially TLR4[18]. Their signalling, in turn, activates NK-κB, which is a crucial component for the triggering of the early inflammation and, having its action blocked, contributes to a reduction in the ischemia/reperfusion injury[18]. The complement system, consisting of a cascading series of plasma enzymes and proteins, is activated through pro-inflammatory cytokines, released by the damaged tissue and capable of inducing the synthesis of some acute-phase proteins in the liver, like C-Reactive Protein (CRP)[17]. Complement complexes are then produced and deposited in the myocardial fibres of the infarction area[17]. The complement is equally involved in the chemotaxis of neutrophils[17].

During the acute period of the MI, apoptosis may be prevented by the hypoxia-inducted expression of B-cell lymphoma-2 (Bcl-2)[17]. Nevertheless, during the course of the infarction there is an expression of promotive (Bax) protein, inductor of the programmed cell death[17]. Some cytokines like Tumour Necrosis Factor (TNF) may also be involved in the activation of this process, as well as oxygen species through the mitochondrial alterations they promote[17]. TNF is equally thought to be involved in a protective effect after an ischemia/reperfusion injury, similarly Interleukin-1β (IL-1β)[18].

The inflammatory cell stage starts when neutrophils adhere to the Endothelial Cells (ECs) in a process called 'rolling-sticking', mediated by adhesion molecules expressed at the surface of neutrophils, such as L-Selectin (functional during the 'rolling' in non-activated neutrophils) and β_2-integrins (upregulated during the 'sticking' in activated neutrophils)[17]. After adhering, neutrophils are induced by adhesion molecules on the vascular ECs, such as P-Selectin and Intercellular Adhesion Molecule (ICAM), to get trapped in the microvasculature[17]. Afterwards, some factors like platelet-activating factor associated with ICAM and other adhesion molecules induce the migration of

neutrophils into the extravascular compartment[17]. Neutrophils may secrete several factors, such as TXA_2 and leukotriene B4 (both responsible for platelet aggregation and vasoconstriction)[17], oxidants and inflammatory cell recruitment mediators[18], as well as Transforming Growth Factor Beta (TGF-β), which stimulates the growth of fibroblasts and neovascularisation, inhibiting acute inflammatory responses after reperfusion techniques[17]. The contradictory effects of neutrophils might be related to their stage of activation, since they release toxic products when they are stuck to the vascular wall, and do not release such products in the evasion stage[18]. Neutrophils are also responsible for the phagocytosis of cell debris and dead cells[18].

2-3 days after the MI, monocytes and macrophages are also found in the ischemic tissue[17]. The migration of these kind of cells is linked to the chemokine expression profile over time, since chemokines are chemoattractants[18]. For example, RANTES (a type of CXC chemokines) and Interleukin-8 (IL-8, a type of CC chemokines) are responsible for the neutrophil and monocyte migration, respectively[18].

The degradation of released macromolecules and dead CMCs is done by mononuclear phagocytes, while Ly-6C(hi) monocytes have phagocytic, proteolyc and inflammatory functions, and Ly-6C(lo) monocytes promote myofibroblast accumulation, angiogenesis and deposition of collagen[18].

Once the reperfusion treatment is started, there is an increase in the production of ROS, capable of triggering a chain reaction that might culminate in the peroxidation of cell membrane lipids and consequent loss of fluidity and changes in permeability[17]. Through NK-κB, ROS may lead to the release of cytokines and chemokines[18]. These oxygen species, produced by neutrophils, also participate in the repair mechanisms of cells by stimulating the proliferation of fibroblasts[17; 18].

I.2.2.1.4 Fibrosis

Fibrosis is a mechanism by which the heart eliminates the necrotic tissue, produced in a short period and resulting from the ischemic aggression, and repairs itself with a scar, based on collagen[19]. This stage follows the inflammatory one, and requires the cessation of cytokine release, removal of inflammatory proteins, and initiation of the collagen production, in order to give rise to the scar tissue[18]. In the beginning of fibrosis, neutrophils become apoptotic cells and[19], unlike necrotic cells, stimulate the production of anti-inflammatory cytokines, such as Interleukin-10 (IL-10) and TGF-β[18]. The latter not only diminishes the adhesion of leukocytes, but also induces the proliferation of fibroblasts and the production of Extracellular Matrix (ECM)[18].

The scar formed by the deposition of fibroblasts and ECM impairs the function of the heart by increasing its stiffness, damaging the mechanic-electrical coupling of CMCs, and increasing the risk of arrhythmias, inducing the CMCs hypertrophy, and ultimately leading to cardiac failure[20]. Despite all the health issues associated, the fibrotic mechanism is the best and fastest answer a human heart, with little regenerative capacity, can give to a fulminant ischemic episode[19].

I.2.2.1.5 Cardiac Remodelling

After an AMI, there is a series of changes in size, shape and function of the heart called cardiac remodelling[21]. One of the manifestations of this process is the cardiac hypertrophy, characterized by

the enlargement of the heart in response to elevated workload or injury[21]. Although the initial effect is beneficial, with a reduction of the wall stress and a maintenance of the output, long-term hypertrophy may lead to a decline in the ventricular function and ultimately induce heart failure[21]. This switch from a compensatory mechanism to the failure of the myocardium involves molecular and cellular events, such as changes in proteins expression affecting the electromechanical coupling, CMCs growth, changes in the energetic and metabolic state of these cells, as well as death of CMCs, and modifications of the ECM[21].

I.2.2.2 Definition and Diagnosis

The definition of a MI must be precise and accepted worldwide, since this disorder is an outcome measure in several studies, such as clinical trials, quality assurance programmes and observational studies[22]. In 2000, a committee composed by experts from the European Society of Cardiology and the American College of Cardiology Foundation proposed a definition of the disorder[23], which was updated as a consequence of the evolution of the assays for myocardial necrosis markers[22]. The most recent definition of the disorder is, therefore, the Third Universal Definition of Myocardial Infarction, approved by the American College of Cardiology Foundation, the American Heart Association, the European Society of Cardiology, and the World Heart Federation, and also accepted by the World Health Organization and the medical community[22].

An AMI is diagnosed based on a specific group of criteria comprising cardiac biomarkers, symptoms of ischemia, modifications of the Electrocardiogram (ECG), and imaging evidences[22].

I.2.2.2.1 Biomarkers

The biomarkers associated with the detection of a MI are the cardiac Troponin (cTn) and the Creatine Kinase MB isoform (CKMB)[22]. An increase in the concentration of any of these biomarkers is defined as a value that exceeds the 99th percentile of a normal reference population, called the Upper Reference Limit (URL), and should be seriously considered as an indicative of an AMI, if occurring simultaneously with some of the aforementioned criteria[22].

Troponin is a protein formed by three subunits, called C, I and T, which constitutes the thin filament of the striated muscle[24]. In the MI context, the most important forms of cTn are I and T because they present a distinctive amino acid sequence from the correspondent troponins of the skeletal muscle, which allows for the creation of monoclonal antibodies highly specific to cardiac troponins[25; 26]. The level of these proteins in the blood rapidly increases 2 to 4 hours after an AMI[27], and persists for many days, since the massive destruction of CMCs releases them into the bloodstream[24]. Despite indicating the necrosis of CMCs, these markers do not reveal the cause for such event, and so they can be present in the bloodstream due to apoptosis, necrosis, turnover of the myocardial cells or increased cellular wall permeability, amongst others[22]. To measure the blood concentration of cTn, blood samples are taken on the first assessment and 3-6 hours later, being important to observe a rising and/or falling pattern on such concentrations to make the distinction between an acute and a chronic elevation of the markers, since chronic elevations might be due to diseases other than AMI, like renal failure[22].

Besides their high myocardial tissue specificity[25; 26; 28], they are also highly sensitive, reaching a sensitivity of 100% compared to only 82% for CKMB[29], which makes cTn I and T the preferred biomarkers for the diagnose of an AMI[22; 28].

Creatine Kinase (CK) is a dimeric enzyme, synthesized in the cytosol of myocytes, responsible for the phosphorylation of creatine[30]. In the heart, the CK has a M and a B subunits, being called CKMB[30]. This biomarker is the best alternative to the cTn, being more robust in clinical specificity for irreversible injury, although less tissue specific when compared to cTn[28; 31], since it is also present in skeletal muscle, in a proportion of 1% to 3%[30]. CKMB should be measured by mass assay every 12 hours, since it is released to the blood stream between 6 to 10 hours after the infarction episode, reaches a peak after 24 hours, and goes back to the normal values 36 to 72 hours later[30].

I.2.2.2.2 Symptoms

The symptoms associated with an AMI are the same as ischemic ones, namely dyspnoea[22], *i.e.* difficulty or pain in breathing[32], fatigue, and discomfort at the level of the chest, upper extremity, jaw bone or upper abdomen, both during exercise or rest, lasting for more than 20 minutes[22]. This discomfort may also be not localized, positional, nor affected by movement of the region[22]. The patient may also suffer from other symptoms, such as nausea, diaphoresis or syncope[22], which are, respectively, the feeling of wanting to vomit, excessive sweat, and brief loss of consciousness[32]. Although associated with an AMI, the fact that these symptoms are not specific for this disorder may, only by themselves, indicate another diagnostic, which points to the importance of taking into account other criteria for the correct diagnose of the disease[22].

An AMI may be asymptomatic or have atypical symptoms, such as palpitations and cardiac arrest[22], *i.e.* the awareness of the fast or irregular beating of the heart, and the stopping of the heartbeat, respectively[32].

I.2.2.2.3 Modifications of the Electrocardiogram

Performing an ECG the moment the patient is observed is a crucial step, not only to observe possible changes in the waves of the cardiac trace but also to have a record for posterior comparison[22]. If the first record is non-diagnostic but the patient presents symptoms, the ECG should be repeated in intervals of 15 to 30 minutes or performed through a constant monitoring, using 12 leads[22]. The changes usually associated with an AMI involve the ST-T and Q waves and might be helpful in the determination of the time the event started, the identification of the infarcted artery, the estimation of the amount of myocardium at risk, the statement of the prognosis, and the establishment of the therapeutic strategy[22]. Since some disorders like pericarditis or left ventricular hypertrophy may also alter the ST wave, the ECG is insufficient to do a correct diagnostic and therefore should be used in conjunction with another diagnostic tool[22].

The J point is the point at which the potential of the ECG is null and is localized at the end of the QRS complex[33]. It varies with age and gender[22]. Taking this into account, an AMI is diagnosed if there is a new ST elevation at the J point in two contiguous leads of at least 0.1 mV in all the leads other

than V_2 and V_3, where the increase must be equal to or higher than 0.2 mV for men at the age of 40 or older, 0.25 mV for men under 40 years, or 0.15 mV for women[22].

I.2.2.2.4 Imaging Evidences

In the context of diagnosing an AMI, non-invasive imaging techniques used must be capable of estimating some parameters, such as perfusion, myocyte viability, myocardial thickness, thickening and motion, and the effects of fibrosis on the kinetics of contrast agents[22]. The most popular ones are mentioned below, with reference to their main utility.

The ECG, already explored in the previous section (I.2.2.2.3), is very useful to assess the structure and function of the heart, specifically the myocardial thickness, thickening and motion[22]. It might also be used to evaluate the myocardial perfusion and microvascular obstruction by use of contrast agents, and to quantify the global and regional function by tissue Doppler and strain imaging[22].

The radionuclide imaging uses radionuclide tracers, such as thallium-201, technetium-99m MIBI, tetrofosmin, F-2-fluorodeoxyglucose and rubidium-82, to directly assess viability and perfusion, although it has a poor spatial resolution, which is a disadvantage when it comes to the detection of small infarct sites[22].

The magnetic resonance imaging allows the assessment of the myocardial function due to its high tissue contrast[22]. The myocardial perfusion and fibrosis might also be evaluated using paramagnetic contrast agents, even when detecting small areas of infarction[22].

I.2.2.3 Classification

The most common categories of AMI are the ST Elevation Myocardial Infarction (STEMI) and the Non-ST Elevation Myocardial Infarction (NSTEMI), based on the respective presence or absence of a ST elevation, in accordance with the previously stated reference parameters[22]. This disorder is further classified as Q wave MI or non-Q wave MI, depending on whether the ECG shows Q waves or not, or even unstable angina when the biomarkers do not exceed the 99th percentile of URL[22]. Beyond these categories, a MI might also be classified as type 1 – type 5, according to the pathological, clinical and prognostic differences, and treatment strategies[22].

I.2.2.4 Epidemiology

Being the leader cause of mortality worldwide, as previously mentioned, CVDs are the source of roughly 4.1 million of deaths annually, in Europe[34]. Its age-standardized death rate per 100 000 members of a population was estimated to be 174.7 for male individuals and 126.8 for female individuals in Portugal during the year of 2011. These can be considered intermediate values when compared to those of 2010 for Spain and the United Kingdom, respectively, 169.7 for male and 110.4 female, and 205.2 for male and 129.0 for female[34].

AMI is a disorder of this group which raises some concern since it is the most relevant cause of CHD morbidity and mortality[35]. Its incidence, which is the number of new cases of a disease per thousand of population in a specific period[32], is generally decreasing, as showed by several studies

around the world[35-40]. In fact, the incidence of a first MI registered a yearly decrease of 2.0-6.0% for male and 1.4-5.9% for female, during the years comprising the gap between 1978 and 2005, in the United Kingdom, Sweden and Denmark[35; 38; 39]. An opposite trend of the incidence of AMI was observed in Japan[41; 42] and Spain[43], while two studies from the United States of America present some colliding results for the evolution of the incidence in both black and white males, as well as both black and white women[44; 45], possibly explained by the different periods considered. In Japan, this increasing incidence rate appears to be connected to the high smoking levels, high rate of aging, adoption of a westernized life style with a diet richer in fat and higher levels of cholesterol, and higher incidence of diabetes and obesity[41; 42]. Some studies have also reported an increase in the incidence of the disorder, of transient nature in some cases, with such effect being attributed to the introduction of a new definition for MI and the use of new and more sensitive biomarkers for the detection of the disease by that time[36; 46; 47]. In fact, with the consensus of 2000, cTn became the preferred biomarker to detect a MI and, as stated in the consensus document, this marker might detect minimal size infarctions[23]. Furthermore, a study comparing the rates of AMI obtained by different cut-off values, the old one, ROC curve (≤ 0.6 μg/L), and the actual one, the 99th percentile of a reference population (≤ 0.1 μg/L) indicated by the consensus of 2000[23], demonstrated an increase in the number of cTn I positive/ CKMB negative cases of 186% when using the 99th percentile instead of the ROC curve[48]. Such increase was, in turn, associated with a rise in the disease incidence, although the extra cases were mild ones[48].

Despite the opposite trend of the incidence for some countries, a consensual decrease is observed in the several mortality rates of the AMI, although of different magnitudes[35; 36; 39-41; 43; 45-47; 49-54]. For example, in the United Kingdom, the 30-day mortality rate registered a decrease of 12.0% in men and 11.0% in women, per year, between 1996 and 2005[35]. On the other hand, Spain showed smaller decreases for the 28-day mortality rate, with the results of 3.7% in men and 4.0% in women, per year, between 1985 and 1997[43]. This reduction in mortality rates might be explained by several factors, such as:

- Better primary prevention of the AMI by controlling the cardiovascular risk factors, namely tobacco smoking, lack of exercise, high blood pressure, and high cholesterol level[36; 39; 40; 43; 45; 46; 49; 50; 52];
- More adequate and improved treatments, making use of thrombolysis, Coronary Artery Bypass Grafting (CABG), and Percutaneous Coronary Intervention (PCI)[36; 39-41; 43; 45; 49-51; 53; 54];
- Enhanced tertiary prevention through the administration of antiplatelet regimens, β blockers, angiotensin converting enzyme inhibitors, and statins[36; 40; 43; 46; 47; 49-51];
- Decrease in the severity of the AMI cases, due to changes in the presentation of the disease, changes in co-morbidities, and higher awareness of the disease[39; 47];
- Higher detection of milder cases of AMI, as a result of the use of more sensitive biomarkers, diluting the number of fatal cases[47].

Some studies have reported disparities between men and women, relating to the trends of the disease. Women who have an AMI are generally 7-11 years older than men[41; 46; 49; 55] and present also

more comorbidities, namely diabetes and hypertension[55]. Some differences in the treatment given to the patients were also reported, with women being less indicated to receive reperfusion procedures and β-blockers, which in turn could be explained by the higher age, and consequent fragility, of women[41; 55]. The referred facts may lead to a poorer outcome for women, who have higher mortality rates[41; 43; 55]. One study on the composition of atherosclerotic plaques highlighted the higher cellular fibrous tissue content in female plaques and the higher dense fibrous tissue content in male plaques, in coronary arteries and saphenous veins [56]. Such compositions may contribute to the differences in the CGD pattern observed in men and women[57].

Whether or not gender can be included as a predictor of the AMI mortality, comorbidities and age are accepted as not only the major risk factors for the development of a CVD but also as indicators of a higher AMI case-fatality[36; 41; 49]. In fact, Capewell and colleagues (2000) found an increase of the 30-day case-fatality from 5.1% in people younger than 55 years to 45.5% in people older than 85 years[49]. Therefore, these studies show that age will have a strong effect in the outcome of the patients and, unlike the remaining risk factors, cannot be prevented, which emphasises the demand for better and more efficient treatments for AMI.

I.2.3 Risk Factors

A risk factor is a condition linked to an increased probability of developing a specific disease [12]. Seeing that AMI is a CVD, the risk factors associated with it are the same as those identified for the general CVDs. Cardiovascular risk factors are clustered into two distinct groups[12]. In the modifiable group one can identify several risks associated with the lifestyle of current societies, such as excessive alcohol consumption[58-60], hypertension[61; 62], high levels of serum cholesterol[63; 64], diabetes[65-67], unhealthy diet[68-71], obesity[72], lack of physical exercise[73; 74], smoking[59; 75], behavioural factors like stress[76-80], amongst others[12]. In the non-modifiable group fit factors that cannot be altered, such as age[81-83], ethnicity[84-86], gender[55; 87; 88] and heredity[59; 89].

The risk of AMI might be reduced by acting on its modifiable risk factors[90]. In fact, according to a study of Slavíček and colleagues (2008), the adoption of a low-fat and low-energy lacto-ovo-vegetarian diet, a light physical training, an abstention of alcohol, smoking, coffee, tea and spicy food, a rest with 8 hours of daily sleep, and a reduction of stress by spending some time out of large cities, contributed to the reduction of several risk factors, such as weight and body mass index, blood pressure, serum cholesterol and blood glucose[90]. In general, a Mediterranean diet, with a moderate consumption of alcohol and salt[70; 71], some exercise[74], and the cessation of tobacco consumption[59; 75] are important steps to reduce body weight, serum cholesterol levels, blood pressure and, therefore, the risk of AMI. Taking action regarding these modifiable risks is the best option to reduce the burden of non-modifiable risks, such as family history[89].

Despite the generalized reduction in the burden of AMI or CVDs, especially in the developed countries[34-40; 45-47; 49-54], there are two things that must be taken into consideration. First, such trend is being counteracted in developing countries and countries adopting a more westernized lifestyle[41; 42; 91]. Second, diabetes[92], blood pressure[93], obesity[94], and aging[7] are conditions with a crescent burden and, therefore, the decreasing tendency for the incidence of AMI in some countries might be reverted[38; 46;

[47]. Taken together, these facts strengthen the importance of acting towards the reduction of the risk factors for CVDs.

I.2.4 Management

After identifying the symptoms and confirming the diagnosis through the methods described in I.2.2.2, and once the patient is no longer in risk of dying, care must be provided in order to reduce the discomfort and pain of the patient[31]. Then it is important to act quickly, restoring the blood flow in the shortest time possible and avoiding recurrent ischemic events[15]. Afterwards, the end-points of the management are to minimize the consequences of myocardial necrosis and avoid future ischemic events[15].

I.2.4.1 Emergency Care

During a MI, patients usually feel a chest pain which should be diminished to avoid the stimulation of the sympathetic system, responsible for the vasoconstriction and increase of the workload of the heart[31]. To accomplish that, morphine or diamorphine might be used in certain dosages and intervals and, in case of failure, β-blockers and nitrates are an alternative[31]. The anxiety of the patient must also be reduced and, if such is not achieved, the patient may receive a tranquiliser[31]. For breathless, heart failure or in shock patients, the administration of oxygen or ventilator support might be necessary[31].

I.2.4.2 Early in-Hospital Care

On average, patients wait around 1.5-2 hours after the symptoms onset to look for medical care[95]. Since the delay in the treatment might increase the mortality rate, patients might be advised to take aspirin and nitroglycerin to initiate a pharmacologic reperfusion therapy[95]. After becoming aware of the AMI symptoms, patients should be transported to the hospital by ambulance to avoid extra delays when reaching the emergency service and to receive any health care on the travel, if necessary[95]. The time factor in this situation is crucial since a fast reperfusion might avoid the necrosis of the myocardium, therefore leading to a greater prognosis[16].

I.2.4.2.1 Pharmacologic Reperfusion Therapy

The pharmacologic reperfusion therapy is a crucial therapy in the treatment of these patients, encompassing fibrinolytic, antithrombotic and antiplatelet agents[96].

Amongst the fibrinolytic agents, streptokinase is one of the most popular, although other agents like tissue Plasminogen Activator (tPA) and its congeners, Tenecteplase tissue Plasminogen Activator (TNK-tPA) and reteplase Plasminogen Activator (rPA), are also used[96]. The name of these agents arises from their fibrinolysis action, which is the enzyme-mediated dissolution of the blood clot, made of blood cells and a matrix of fibrin[97]. Basically, tPA or streptokinase activate, through distinct processes, the plasminogen in circulation, converting it into plasmin, which will then do the process of fibrinolysis[97].

To sustain the action of the fibrinolytic agents[97] and prevent the thrombosis phenomenon, including acute thrombosis during PCI, antithrombotic agents are used, such as unfractionated heparin (which contributes to the anticoagulation effect by inactivating thrombin and the activated factor X[98]), low molecular weight heparin, activated factor X inhibitor (which acts in the coagulation cascade and prevents the generation of thrombin), and direct thrombin inhibitor (which blocks the activity of thrombin, factor IIa, preventing the conversion from fibrinogen to fibrin)[99].

The aim of the antiplatelet agents is to avoid platelet aggregation, induced by the increased concentration of free thrombin that follows the action of fibrinolytic agents[14]. Aspirin is used in this context, as well as clopidogrel[15] and platelet glycoprotein IIb/IIIa receptor blockers, much stronger inhibitors than aspirin due to their action in the final common pathway of the platelet aggregation, instead of acting in only one pathway as aspirin does[14]. Aspirin acts by inactivating the platelet cyclooxygenase through an acetylation in its active site, which prevents the production of TXA_2 by the platelets[100]. Without this protein, the aggregation of the platelets is diminished[100].

The fibrinolytic therapy should be administered within 30 minutes of patient's arrival to the emergency service if a PCI cannot be performed instead within 120 minutes[95]. This therapy should also be accompanied by the administration of an antiplatelet and antithrombotic agent for varied periods[95].

I.2.4.2.2 Percutaneous Coronary Intervention

One of the most used and effective techniques in the management of AMI patients is PCI, also known as angioplasty, Percutaneous Transluminal Coronary Angioplasty (PTCA) or balloon angioplasty[99]. It basically consists in the re-establishment of blood flow in a blocked coronary artery through the inflation of a balloon inside the vessel[99]. Other devices or techniques might be used together with PCI, namely a stent or drug eluting stent, to keep the lumen of the vessel unobstructed, a rotational or laser atherectomy or even a brachytherapy, where a radioactive source inhibits the restenosis of the vessel[99].

A primary PCI without fibrinolytic therapy is advised for patients with high-risk features like shock, high risk of bleeding induced by the pharmacologic reperfusion therapy, late arrival at the urgency service (exceeding 3-4 hours of the symptoms onset), and short transfer times to PCI-capable hospitals in case of a presentation at a non-PCI-capable one[95]. The technique must yet be applied within 90 minutes of the patients' presentation at the urgency service and in the case of fibrinolytic therapy failure[95]. A delayed PCI might also be considered in the case of a significant stenosis in patients who received a successful fibrinolytic therapy, being applied as soon as possible within 24 hours, but never in less than 2-3 hours after the fibrinolytic therapy[95]. Unlike fibrinolytic therapy, primary PCI is associated with higher rates of infarct artery patency, TIMI 3 flow, and access site bleeding, and lower rates of ischemia recurrence, reinfarction, revascularization procedures repetition, intracranial hemorrhage, and death[95]. The administration of an antiplatelet and antithrombotic therapy is recommended before, during and/or after PCI as a supportive measure[95].

I.2.4.2.3 Coronary Artery Bypass Grafting

CABG is a surgical procedure performed under general anaesthesia and requiring a median sternotomy (a surgical procedure where the breastbone is cut, allowing a heart operation[32]), in which a vessel from another region of the patient's body is implanted to make a bypass of the atherosclerotic narrowings in the coronary artery[99]. The most common vessels used in this procedure are the saphenous vein, the internal thoracic artery and, recently, the radial artery[99]. This therapy is advised for patients who are not amenable to undergoing PCI or fibrinolytic therapy or in whom PCI failed[95].

I.2.4.2.4 Heart Transplant

Unlike the previously described therapies, a cardiac transplantation gives a solution to the loss of healthy CMCs[101], thus being an acceptable treatment for heart failure[102]. The first cardiac transplant was performed by Dr. Christiaan Barnard in a 54-years-old man who had a very damaged heart due to repeated MI, on 3rd December of 1967, South Africa[103]. Although the postoperative exams were satisfactory[103], the patient died 18 days later with an infection by *Pseudomonas pneumonia*[104]. Ever since, the cardiac transplantation has been performed and improved, in order to offer a longer and better quality of life to the patients[104].

To successfully transplant a heart, patients must be selected with caution, usually corresponding to those who do not respond to the conventional therapies[105]. A match of body size and blood group is also required between the receptor and the donor[105]. After the transplant, the patient has to follow a rigorous immunosuppressive drugs treatment to avoid the rejection of the graft[105]. A heart transplant may not only offer higher and longer survival rates than medical treatment only[102], but also a significant improvement of the physical condition and life quality[102; 106]. Nevertheless, this therapy is still associated with the need to take immunosuppressive drugs and the risk of graft failure, heart failure, infections and malignancies[102; 107]. The amount of cardiac donors is also a limiting factor that hinders the transplant in a higher number of patients[107].

I.2.4.3 Long-Term Care, Secondary Prevention and Rehabilitation

In the long-term care there are some important drugs to avoid any ischemic occurrence and minimize the damage and impairment induced by the AMI[14].

Aspirin should be continuously and indefinitely administered as an antiplatelet treatment to avoid reinfarction and stroke[14]. Anticoagulant agents can be paired with aspirin to avoid the formation of thrombus and to reduce the mortality rate and the risk of reinfarction and stroke, although it is necessary to take into consideration the risk of bleeding complications[14].

β-blockers are advised in cases of tachycardia without heart failure, hypertension and chest pain that does not diminish with opioids, and might be administered for an undetermined period[14]. Basically, this agent induces a decrease in heart rate, blood pressure and myocardial contractility, leading to a reduction in the oxygen by the myocardium[100]. It also exerts a beneficial effect in the distribution of myocardial blood flow and prevents the arrhythmogenic action of catecholamines[100]. All the effects combined lead to a reduction of the infarct size, myocardial wall stress and recurrent ischemia, and prevention of cardiac rupture[100].

Calcium channel blockers may be an alternative when β-blockers are not tolerated by the patients[95]. These agents decrease blood pressure and myocardial contractility, contribute to the dilatation of coronary arteries and prevent calcium overload of ischemic cells, thus reducing the oxygen demand by the cardiac muscle[100].

Angiotensin-Converting Enzyme (ACE) inhibitor is another important drug which should be indefinitely administered to reduce the rate of reinfarction and counteract the development of congestive heart failure[14]. This protein inhibits the conversion of inactive angiotensin I to active angiotensin II, and with lower blood levels of the latter, the tension within the wall of the left ventricle decreases through a decrease in the afterload and preload[100]. Such tension reduction leads to a smaller oxygen demand[100].

Nitrates act by inducing the dilatation of venous capacitance vessels, arterial resistance vessels, and coronary arteries, also redistributing the blood flow to ischemic areas[100]. This agent reduces afterload and preload, thus diminishing the wall stress of the myocardium and, therefore, the oxygen consumption[100].

Statins are important to control the concentration of lipids like cholesterol in circulation[95].

Beyond the medication that must be taken, patients should also follow a healthier lifestyle, through the adoption of a Mediterranean diet, control of body weight, cessation of tobacco consumption, and practice of physical exercise, as a secondary prevention of new ischemic episodes[31].

For patients suffering from a significant left ventricular dysfunction, rehabilitation is strongly advised in order to allow the patient to return to work and have a life as normal as possible [31]. The outcome of rehabilitation may be improved by reducing the emotional distress to which the patient is subjected throughout the AMI episode and respective treatment[31]. The acquisition of a physical exercise routine is also beneficial for the recovery and for reducing the mortality risk, but should be adjusted to the age, pre-infarction level of activity and physical limitations of the patient[31].

I.2.4.4 Non-conventional Therapies

Non-conventional therapies for AMI encompass protein therapy, gene therapy and cell therapy[99]. In the protein therapy one can highlight the Heat Shock Protein 27 (HSP27), a chaperone that is thought to act by inhibiting the caspase cascade, thus protecting the CMCs under apoptotic stimuli[99]. Other proteins such as angiogenic growth factors and anti-apoptotic fusion proteins have been explored aiming at the neovascularization and the apoptosis in infarcted hearts, respectively[99]. An example of Gene Therapy includes the injection of angiogenic genes in plasmids or adenovirus[99]. Due to the scope of the present thesis, cell therapies will be further explored in the next chapters.

I.3 Cardiac Regeneration and Repair

I.3.1 Intrinsic Regenerative Potential

Contrary to what is observed in newts[108] and zebrafish[109], the heart of humans and other mammals has been seen as a terminally differentiated organ, without the capacity to regenerate loss CMCs[110]. In fact, during the fetal stage, CMCs have a hyperplastic growth, based on a high

proliferation capacity, which is lost after birth[111]. Before losing this proliferative potential, these cells perform one last incomplete cell division and become binucleated CMCs[111], as shown in a study with rats[112]. Any growth the heart presents after birth is therefore obtained by a hypertrophic process and might be stimulated either by physical exercise, pregnancy or even pathologic stimulus[113]. Cardiac hypertrophy maintains or augments the pump function by decreasing the stress of the ventricular wall, being associated with the energy obtaining through a balance of both glycolytic and fatty acid oxidation in the case of exercise, and a switch to the glycolytic path in the pathological case[113].

This absence of proliferation in the adult life and consequent lack of regenerative capacity might be associated with the high-pressure circulatory system, present in human and other mammals, which is incompatible with a long duration bleeding and therefore requires a fast healing, only achieved through fibrosis[114]. This is not the case in zebrafish and newts, where the circulatory system is a low-pressure one[114]. Besides this difference, others might be highlighted, such as the reduced presence of fibroblasts in the heart and an incomplete adaptive immunity of these animals[114]. Altogether, these features contribute to the cardiac regenerative response to injury, which takes approximately 30 and 60 days in newts[108] and zebrafish[109], respectively, unbearable times for the human case[114].

The importance of having an immature immune system for the regeneration of the heart is supported by some studies, namely one conducted by Blewett and another conducted by Haubner. In 1997, Blewett and co-workers studied the regenerative capacity of fetal mouse hearts and observed that 14-days-old hearts were capable of healing without signs of inflammatory response or collagen fibre deposition, as a result of CMCs migration to the injured site[115]. However, for 18 and 22-days-old hearts, authors observed an incomplete healing of the heart without collagen deposition, or a wound healed by scarring with collagen deposits, respectively[115]. By the 18th day, the migration of CMCs was not observed, since these were already too differentiated[115]. Instead, fibroblast migrated to the wound and formed the scar in the older hearts[115]. Haubner and colleagues (2012) successfully demonstrated the full heart regeneration in newborn mice that suffered severe cardiac ischemia[116]. This ability, lost at the 7th day after birth, was partially attributed to CMCs, at the edge of the injured site, which re-entered the cell cycle and started proliferating[116].

I.3.2 Evidence of Human Heart Regeneration

Studies carried out near the beginning of the century have shown that, after all, the adult heart still has some regenerative potential, albeit at a reduced degree. In 1998, Kajstura and colleagues conducted a study with 27 patients undergoing cardiac transplantation and 9 control hearts, which revealed a small percentage of CMCs experiencing a mitosis process in the left ventricle, both in the normal and pathological hearts, of around 0.0014% and 0.014%, respectively[117]. Besides demonstrating that proliferation was still possible, the study evidenced a 10-fold increase of the proliferation in injured hearts, compared to normal ones[117]. Later on in 2001, a study by Beltrami and co-workers with adult patients having infarcted hearts showed the presence of myocytes, not terminally differentiated, that entered the cell cycle to produce CMCs, early after infarction[118]. Authors estimated that within 30 minutes mitosis process and 520 myocytes/million of CMCs undergoing cell division (identified by Ki-67, a marker of cell proliferation), the CMCs lost due to the infarction episode

(around 1.70 x 10^9 in the left ventricle) could be replaced after 18 days[118]. Thus, there was evidence of a turnover rate of CMCs throughout life[118].

Taking advantage of the incorporation of ^{14}C, generated during the Cold War, into de Deoxyribonucleic Acid (DNA), Bergmann and colleagues (2009) found evidence of DNA synthesis many years after birth, in humans[119]. With a turnover rate declining with age (from approximately 1% at the age of 25 to 0.45% at the age of 75), authors also estimated that by the age of 50, 40% of the CMCs, produced by an unknown source, would have been generated after birth[119], a renewal rate much smaller than that estimated by the group of Beltrami (2001) of approximately 100% new CMCs generated after only 5 years[118].

With evidences pointing to the loss of hyperplastic growth capacity after birth[112] and, oppositely, still able to undergo mitosis especially when subjected to a MI[117; 118], researchers started wondering about the origin of sudden cycling myocytes[120]. Orlic and colleagues (2001) reported a population from the Bone Marrow (BM), Lineage-negative (Lin^-) and c-kit^{POS}, capable of differentiating into CMCs, ECs and smooth muscle cells, and improving the heart function when injected directly into the contracting wall bordering the infarct of the heart in transgenic mice expressing enhanced green fluorescent protein[121]. Quaini and colleagues (2002) proved the occurrence of cardiac chimerism in female human hearts transplanted into male receptors who suffered large myocardial damage[122]. Basically, the presence of Y chromosomes was detected in 18% of the myocytes, 20% of the coronary vessels, and 14% of capillaries in the female transplanted heart, which has only X chromosomes[122]. Surface markers of stem cells, namely c-kit, Multidrug Resistance Protein 1 (MDR1) and Stem Cell Antigen-1 (SCA-1), were used to identify positive cells in the atria and left ventricle of the transplanted hearts[122]. In 2004, cardiac chimerism was confirmed in patients receiving a BM or Peripheral Blood Stem Cells (PBSCs) transplant of a gender-mismatch donor[123]. Laflamme and colleagues (2002) also observed the chimerism phenomenon[124], but in a much lower rate than that reported by the group of Quaini (2012)[122]. In fact, only 0.04% of the CMCs present in the transplanted heart were of recipient origin[124].

I.3.3 Evidence of Cardiac Stem Cells

The aforementioned studies, indicating the possibility of stem cells being responsible for the generation of CMCs, triggered the arise of several other studies describing different cell populations which could, in fact, correspond to true stem cells.

In 2003, Beltrami and colleagues isolated, from rat hearts, a population of Lin^- c-kit^{POS} cells with the features of a stem cell population, namely self-renewing, clonogenicity, and multipotency[120]. Upon injection, these authors were also capable of migration towards the ischemic zone, regeneration up to 70% of the left ventricle myocardial wall, and improvement of the pump function of the heart[120]. The study could not, however, certify the origin of these cells, since these could be either cardiac cells or cells from other organ which previously migrated to the heart[120]. In addition, a resident myocardial stem cell-like population was identified in rats through the use of Hoechst dye, by Hierlihy and co-workers (2002)[125]. Extracted from the ventricle, these Side Population (SP) cells (whose name derived from the characteristic efflux of a Hoechst) represented 1% of all the cells in the heart and originated myotubes when co-cultured with primary-derived Skeletal Myoblasts (SKMs) and H9C2 rat CMCs,

although it was not clear if the creation of new CMCs resulted from cell fusion[125]. The existence of a cardiac SP in mice was later on confirmed by the group of Pfister (2005), who further refined the cardiac progenitor population as lacking the expression of the Cluster of Differentiation 31 (CD31) but expressing Sca-1 and being cardiac SP, and highlighted the importance of the co-culture with CMCs for these cells to be able to differentiate into mature CMCs[126].

In 2003, Oh and colleagues obtained a Sca-1$^+$ cell population lacking the marker c-kit and hematopoietic lineage markers such as CD34 and CD45[127]. When injected in an induced ischemia/reperfusion injured mouse heart, these cells engrafted in a persistent way after 2 weeks and differentiated into CMCs, although cell fusion accounted for 50% of the newly generated cells[127]. Around the same time frame, the group of Messina (2004) was capable of isolating a mixture of cells, which they called cardiospheres, both from humans and mice[128]. These cardiospheres were self-renewing, had spontaneous beating (in the case of human cardiospheres, co-culture with rat CMCs was necessary to observe such phenomenon), and a high expression of c-kit and several other markers associated with endothelial and stem cells, thus showing evidence of their stem/progenitor cell profile[128]. The human cardiospheres were also capable of differentiation, *in vitro* and after ectopic (dorsal subcutaneous connective tissue) or orthotopic (MI) transplantation in Severe Combined Immunodeficient (SCID) beige mouse, into cells with contractile activity and/or CMCs markers and cells with endothelial and smooth muscle markers[128].

The group of Laugwitz described, in 2005, the presence of a cell population positive for the LIM-homeodomain transcription factor islet-1 (isl-1) in the human, mouse and rat heart[129]. This population, distinct from the previous potential Cardiac Stem Cells (CSCs, c-kit$^+$, Sca-1$^+$ and SP cells) due to the absence of the respective markers, was found to be responsible for the generation of 30-40% of CMCs during mouse embryonic development[129]. The differentiation into mature CMCs was cell-dependent but not resultant of cell fusion[129].

In 2007, the group of Bearzi successfully identified human CSCs has being positive for c-kit and negative for hematopoietic markers, and capable of differentiating into CMCs, smooth muscle cells and ECs[130]. When injected into infarcted mice or rat, these cells were capable of giving rise to human myocardium, including blood vessels, without signs of cellular fusion[130]. After their implantation, the Ejection Fraction (EF) and ventricular function improved, while the chamber dilation diminished[130].

Limana and colleagues (2007) identified two distinct populations of cells residing in the epicardium (inner layer of the pericardium, a membrane that surrounds and supports the heart[32]) of both human and mouse, called Epicardially Derived Cells (EPDCs), with one being positive for c-kit and the other being positive for CD34[131]. After a MI, the number of mouse c-kit$^+$ cells in the epicardium increased, and their differentiation into a myocardial, endothelial, and smooth muscle phenotype was induced[131]. Regarding the human populations of c-kit$^+$ and CD34$^+$, both expressed the early marker of cardiomyocyte differentiation, Nkx2.5, and the cardiac transcription factor GATA4, being also capable of acquiring an endothelial phenotype *in vitro*[131].

More recently, in 2013, Ellison and co-workers showed that a particular population of mice CSCs characterized by being c-kit$^+$ CD45$^-$ tryptase$^-$, quiescent in normal situations, was activated upon a myocardial injury (promoted with a high dose of isoproterenol, a drug that spares CSCs but

kills CMCs) and started proliferating[132]. After proliferation, these cells were able to differentiate into CMCs[132]. The authors proved not only that the new CMCs were not from cycling CMCs, but also that their origin was not BM stem or progenitor cells, nor even cell fusion[132]. By the end of the replacement of the lost CMCs, the stem cell population diminished in number and went back to the quiescent state, to ensure myocardial homeostasis[132].

I.4 Cell Therapy

As several studies have shown throughout the last decade, the heart has, indeed, a regenerative potential that is, nevertheless, insufficient to replace the CMCs lost due to an AMI[119]. However, this humble potential might be explored in order to create a therapy capable of addressing the gaps that conventional therapies for AMI present, such as the absence of replenish of dead CMCs[119]. Such a therapy might be achievable through stem cells or their differentiated progeny, alone or associated with adequate drugs[119].

I.4.1 Definition of Stem Cell

The definition of a stem cell and its features has been a matter of discussion since the beginning of their study[133]. With an already established set of criteria for their definition, some aspects such as the experimental limitations that might hamper the verification of all these criteria, the alteration of the potential of a cell throughout time and according to certain stimuli, and the different relevance of the criteria, may interfere or even bias the classification of a cell as a stem cell[134]. Notwithstanding, the criteria set generally accepted to define a cell as being a stem cell is the following:

- Clonogenicity – capacity of the cell undergoing a mitotic process to originate a large number of progeny, also called clone of cells[134];
- Self-maintenance – sometimes also called self-renewal, it involves the maintenance of the state/potential of the cell and the number of stem cells in a specific tissue[134; 135]. Since progenitor cells also present this feature, stem cells are distinguished by a self-renewal of long term, that is, throughout lifetime[135];
- Potency – ability to produce differentiated cells, which are cells that acquired a certain gene expression pattern that makes them functionally competent and morphologically distinct[134; 136].

Adult Stem Cells (ASCs) are multipotent stem cells, *i.e.* they are able to generate cells from only one embryonic germ layer[137]. ASCs are found in almost every adult human tissues or organs and aim to maintain the homeostasis of the tissues or to promote their regeneration in case of injury, by giving rise to new differentiated cells[138]. Although associated with a more limited proliferation and differentiation capacity, these cells lack the ethical issues and the risk of teratoma formation of PSCs, which makes them more suitable for a cell therapy[139]. Other important sources of multipotent stem cells derive from neonatal tissues, such as the umbilical cord, the umbilical cord blood[140], and the placenta[141].

I.4.2 Types of Cells with Potential for Acute Myocardial Infarction Therapy

The discovery of existent mechanisms for cardiac regeneration has motivated the development of therapies to improve that process in case of considerable damage[101]. Amongst the different approaches that have been developed, one of the most relevant is the delivery, to the heart, of either an endogenous or an exogenous stem cell population in order to activate endogenous progenitors, differentiate into CMCs or ECs, promote neovascularisation, inhibit apoptosis, or even lead to a favourable modulation of the ECM[142]. Some of the most important multipotent stem cells with the potential for such a purpose will be briefly explored in the next sections.

I.4.2.1 Cardiac Stem Cells

As presented in section I.3.3, the mammal heart has one or several clusters of endogeneous stem or progenitor cells capable of generating CMCs[120; 126-130; 132], ECs[120; 128; 130; 131], and smooth muscle cells[120; 128; 130; 131].

CSCs not only have a true regeneration capacity[120; 130; 143], but also a paracrine effect upon the remaining cells of the heart[143-145], which contributes to an improvement of the cardiac function[120]. However, their harvesting involves the performance of a biopsy, even if only in a minimally invasive way[146], and their numbers in the heart are reduced[125; 147], which implies a time consuming expansion before reaching a clinically relevant number[148]. Despite their significant number in injured hearts, CSCs display limited natural response to an infarction, which might be explained by an unfavourable environment which is created upon such an episode, with a low oxygen tension, a high oxidative stress, and an inflammatory response[149].

I.4.2.2 Endothelial and Progenitor/Stem Cells

Endothelial Progenitor Cells (EPCs) are $CD34^+$ mononuclear cells that are found in the BM and, upon an ischemia incident, are recruited to the Peripheral Blood (PB), mobilized to the injured tissue, and differentiate into ECs to generate new blood vessels[150]. ECs are the cells that cover the interior of the vessels, controlling the passage of materials and white blood cells into and out of the bloodstream, and regulating the function and structure of the vessels[151].

Several studies regarding EPCs are in agreement about the importance of these cells for the generation of new blood vessels, either by integration[152; 153] or by paracrine factors[153; 154], contributing to an improvement of the cardiac parameters[153; 154]. However, some studies pointed out the decreased availability and performance of these cells in patients presenting cardiovascular risks[155; 156], which might prove to be a handicap when conceiving an autologous therapy for AMI. The probability of these cells being able to generate CMCs is also reduced[157].

I.4.2.3 Hematopoietic Stem Cells

The first study pointing towards the existence of stem cells in the hematopoietic system was conducted by Till and McCulloch, who injected a cells suspension from the BM into lethally irradiated mice and observed the generation of colonies in their spleens[158]. Containing several differentiated

cells of multiple blood lineages, these colonies were termed Colony-Forming Units (CFUs) and their number was proportional to the number of cells primarily injected[158].

Hematopoietic Stem Cells (HSCs) are the cells responsible for the production of mature blood cells, namely erythrocytes, platelets, granulocytes, macrophages (myeloerythroid cells), dendritic cells, T cells, Natural Killer (NK) cells, and B cells (lymphoid effector cells)[159]. It is estimated that every second, 1.5 x 10^6 blood cells are produced in an adult human, a number justified by the short life span of the cells[159]. HSCs are usually in a quiescent state and seldom divide, so the high production of the blood cells is ensured by the more committed cells they generate, the progenitor cells[159]. This exerts a protective effect on HSCs by avoiding gene mutations during cell division and subjection to damage-inducing metabolic side products and ROS, typical of more metabolically active states[159]. These scarce cells might be identified by the expression of CD90 and CD34, and by the lack of expression of Lineage markers, *i.e.* CD14 (monocyte/macrophage), and CD38[159].

HSCs have been shown to help in the neovascularization and regeneration of the myocardium[160], though the produced CMCs are probably a result of cellular fusion[161]. The injection of these cells also contributes to the improvement of the heart function[162].

I.4.2.4 Skeletal Myoblasts

SKMs are the progenitor cells of myofibers[163]. These cells are resistant to ischemia[139] and can be extracted from the patient in an autologous setting, being cultured *in vitro*, usually for 3-4 weeks, in a scalable way[163].

In spite of their great proliferation capacity [163], resistance to ischemia[139], evidence of improving heart function after transplantation[164; 165], and low risk of tumor formation[163], SKMs are not able to differentiate into CMCs nor show an electromechanical coupling with the recipient CMCs[166-168], which might explain the increased risk of arrhythmias associated with their engraftment in the heart[169-171].

I.4.2.5 Mesenchymal Stem/Stromal Cells

Due to the scope of this thesis, MSCs will be further explored in the section I.4.4.

I.4.3 Steps in Implementation of Cellular Therapy

After choosing the type of cell to be used in the therapy, cells are isolated from their source and an expansion step might be necessary[172] in order to achieve a number of cells that has clinical relevance[173]. In fact, the cell dose needed for the therapy varies according to the type of cell chosen and has been explored in some clinical trials[142].

Once purified and expanded, the cells must be delivered to the injured region, in such a way that their retention is maximized[174]. To this end, the main delivery strategies in the cardiovascular setting are:

- Intracoronary artery infusion – cells are delivered through the central lumen of the vessel using a balloon catheter[174]. It allows a higher homogenization of the cells to the site of injury[174]. Nonetheless, the targeting of the injured myocardium requires the cells to migrate out

of the vessels, which is not so efficient then, nor even an intrinsic feature of some types of cells like SKMs[175]. The retention of the cells is also lower than when using other techniques[142].

- Intravenous injection – this technique is simple and minimally invasive, and its successful delivery of the cells to the injured site relies on homing signals, which is somewhat limiting[176]. The efficiency of this delivery method is also low due to the high probability of the cells getting trapped in the lungs or other organs, not reaching the target[176].
- Direct injection in the ventricular wall – is chosen when the first strategy cannot be applied (for example, the coronary artery is occluded, the scar tissue provides few homing signals, or the cells have a size that might induce a microembolization)[174]. Upon injection, the cells are not homogeneously distributed, which may lead to their death, and there is a risk of perforation of the muscle[174]. The injection might yet be transendocardial (the needle catheter crosses the aortic valve and is positioned against the endocardial surface) or transepicardial (performed during a CABG, it is very invasive but allows the visualization of the injury)[174].

The delivery must also be performed within the most adequate time-frame, considered to be between 7 to 14 days after the AMI, since in the early days of the ischemic episode the inflammatory process could hamper the effects of the therapy, and after 2 weeks the therapy is less efficient[177].

The next step is to ensure that the delivered cells survive and engraft in a long-term way, which might be accomplished through heat shock after the transplantation, forcing the expression of survival factors by the transplanted cells[172], exposure of the cells to prosurvival factors[178], and implantation of cells included in scaffolds made of biocompatible matrix[142]. The immune rejection associated with allogeneic settings must also be avoided by administration of immunosuppressors, for example[172]. Altogether, these aspects are crucial to optimize the positive results of the cellular therapy[142].

I.4.4 Mesenchymal Stem/Stromal Cells

I.4.4.1 Definition

The first evidence to support the existence of a distinct stem cell population residing in the BM dates from 1968, when Friedenstein and colleagues found a set of cells that were adherent, clonogenic, nonphagocytic, and fibroblastic in habit, *i.e.*, they were Colony-Forming Unit-Fibroblast (CFU-F)[179]. Since then, several research groups have explored MSCs and their respective properties, which raised the need of establish a consensus on their definition[180]. Aiming towards that, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a minimal criteria set to define this population, based on the adherence to plastic during their culture, expression of a specific cluster of markers, and multipotent differentiation potential[180].

When cultured without differentiation factors, MSCs grow in a layer, attached a surface, and have a fibroblast-like morphology, as it can be seen in Figure I-2[181]. After inoculation at low density (between 100 and 1000 cells/cm^2)[182], these cells form CFU-F[181].

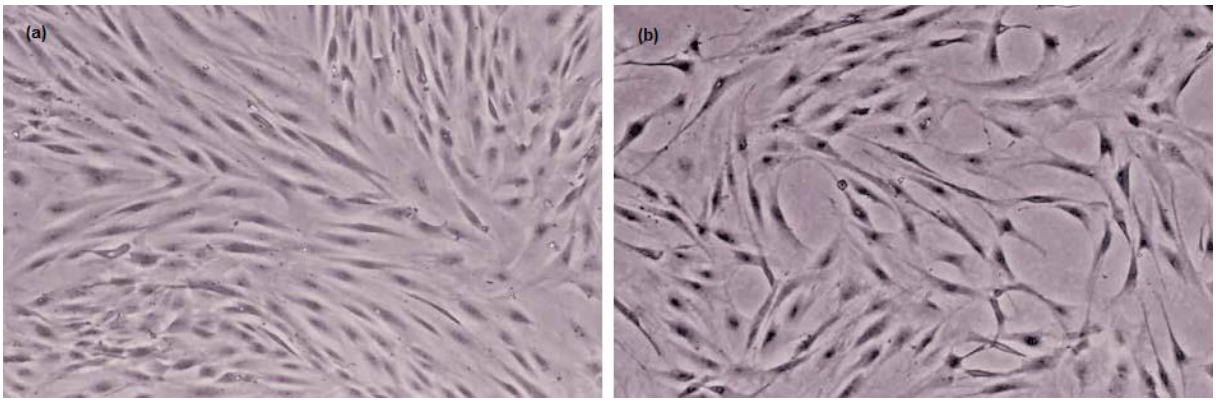


Figure I-2. Human bone marrow and adipose tissue mesenchymal stem cell (MSC) cultures. From (a) bone marrow; (b) lipoaspirate[183].

I.4.4.2 Sources

Human MSCs can be isolated from several tissues. The most popular are BM[179], Adipose Tissue (AT)[184], and, more recently, placenta[185] and umbilical cord structure or blood[186; 187]. Other alternative adult sources of this population or similar ones are synovium[188], cartilage[189], dermis[190], amongst others[181]. During fetal development, MSCs might also be found in the lungs, liver and spleen[191]. Although present in all the referred tissues, MSCs appear to have some differences according to their source, namely in their differentiation potential[192; 193].

BM is the first reported source of MSCs[179] and it is considered to be the gold standard source, despite the low frequency of MSCs, only 0.01-0.001% of nucleated cells[193]. The harvesting of MSCs from the BM may be painful, usually requires anaesthesia, and does not provide a high number of stem cells[184]. This low frequency of Bone Marrow Mesenchymal Stem/Stromal Cells (BM-MSCs) which declines even further with age[193] implies an expansion step *ex vivo*[184]. This step, in turn, is time consuming, expensive, and entails a risk of cell contamination and loss[184]. AT appears to be an attractive alternative to the standard source of MSCs since it is usually discarded as a medical waste from abdominoplasties and lipoaspirations (which became more frequent due to the increased number of obesity cases)[194], more easily accessible through a less invasive method[192; 193], and richer in stem cells when compared to BM[195], which might lead to a reduced period of expansion of cells *in vitro*[196].

The usual procedure to isolate MSCs is based on the adherence of these cells to tissue culture plastic[182]. First it is necessary to isolate the mononucleated cells from the BM aspirate by centrifuging the sample on a density gradient[182]. In the case of the AT aspirates, these need to be firstly processed, usually by an enzymatic method[184], to isolate a heterogeneous cell population called Stromal Vascular Fraction (SVF)[194]. The SVF encompasses adipose stromal cells, HSCs, ECs, erythrocytes, fibroblasts, lymphocytes, monocytes/macrophages, pericytes, amongst others[194]. Upon seeding of mononucleated cells or SVF cells, an adherent population of MSCs or Adipose-derived Stem/Stromal Cells (ADSCs) might be recovered[182].

Other methods might be used to isolate MSCs, for example based on the use of STRO-1 antibody, namely for BM MSCs, which shows the highest affinity and efficiency in the isolation of

MSCs enriched in CFU-F, associated to a Magnetic Activated Cell Sorting (MACS) and/or Fluorescence Activated Cell Sorting (FACS)[182].

Despite some studies pointing to an opposite trend[197-199], Kern and colleagues (2006) showed that ADSCs had lower senescence ratios at early passages, could be cultures for longer periods, and achieved higher cumulative population doublings after P4 when compared to BM-MSCs[200]. The authors also observed the presence of higher numbers of CFU-F in the AT[200]. This was further confirmed by the group of Dmitrieva (2012), who found that the frequency of CFUs isolated from human BM reached a total of 0.0029 ± 0.0008%, while the value was clearly higher for the AT sample, 0.12 ± 0.096%[195]. Peng and co-workers (2008) also presented evidence of ADSCs having greater proliferation capacity and faster population doublings than BM-MSCs[193].

In the view of the differences pointed out between MSCs and ADSCs, one of this work's goal was to perform the comparison of some key properties of MSCs, (namely the proliferation, angiogenesis, secretory profile, and oxidative stress resistance) extracted from BM and AT, to highlight and confirm some similarities and differences and to sustain the hypothesis that AT can be reliable source of MSCs, in alternative to BM.

I.4.4.3 Differentiation Potential

Given that MSCs are multipotent, they are capable of giving rise to a set of differentiated cells from their germ layer, the mesoderm[181], such as osteocytes[201], chondrocytes[202], and adipocytes[203]. These three types of cells compose the classical progeny of the MSC, although other studies have found that this stem cell population is also able to generate other mesoderm cells like myocytes[204], tenocytes[205] and even CMCs[121; 206].

I.4.4.4 Markers

MSCs might be identified through a set of surface antigens that are not specific for this population but rather expressed by other types of cells[181]. Since the cell population is heterogeneous[207] and its antigenic profile suffers alterations throughout their lifespan and has some disparities amongst clones[181], it is unlikely that a universal marker for this population will ever be found[181; 207]. Table VII-1 from Annexes shows the panel of markers that cells must or must not express to be classified as MSCs. The antigens marked (with a *) correspond to the cluster of markers referred by the Mesenchymal and Tissue Stem Cell Committee of ISCT, being part of the minimal criteria to defined this population. The "positive markers" (CD73, CD90, and CD105) must be equal or higher than 95% positive, while the "negative markers" (CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR) must be equal or lower than 2% positive[180].

I.4.4.5 Bone Marrow Niche

The niche is basically a tissue microenvironment[208], comprising all the cells besides stem cells, the ECM, and soluble molecules present in the surrounding of the stem cells[209]. This microenvironment is crucial to give stem cells specific clues that will induce the maintenance of their undifferentiated state, whenever they need to promote the regeneration of a tissue, external clues will

reach the niche and induce their differentiation[209]. One of the key elements of the niche is a group of proteins called cadherins, which are responsible for cell-cell adhesion, migration, differentiation, polarity, and interaction with Wnts (mammalian homologue of Drosophila wingles), which are thought to be involved in both the maintenance of the undifferentiated state and the differentiation paths of the MSCs[209].

The ECM is composed of collagen fibres, laminin polymers, cell adhesion proteins like fibronectin, proteoglycans, and growth factors[210]. In the case of bone, two types of ECM co-exist, the one that is produced by osteoblasts, being therefore calcified, and the one synthesized by MSC, called marrow ECM[210]. The ECM is highly important for the differentiation of MSCs, as shown by Datta and colleagues (2005)[211]. Authors observed that an ECM produced by osteoblastic cells and deposited on a decellularized scaffold induced a higher proliferation of MSCs, as well as a higher deposition of calcium and augmented levels of osteopontin, indicating the differentiation of the stem cells into bone progenitor cells[211].

The BM, localized in the centre and epiphysis of bones, is the local where the hematopoiesis occurs and it comprises a meshwork of thin-walled capillary-venous and ECM[212]. In this structure there is a niche that contains both hematopoietic stem/progenitor cells and the MSCs, as well as adipocytes and stromal cells[210]. MSCs are perivascular and are usually located in the central area of the BM, or less frequently in the vicinity of the endosteum[213]. They are also important supporters of long-term growth of HSCs and hematopoiesis, probably through both the regulation of the immune system and the production of growth factors and chemokines such as Angiopoietin-1, CXCL12, fibronectin, Interleukin-6 (IL-6), Interleukin-11 (IL-11), Leukemia Inhibitory Factor (LIF), osteopontin, Stem Cell Factor (SCF), thrombopoietin, TGF-β, Tumour Necrosis Factor Alpha (TNF-α), amongst others[214; 215]. The BM niche is hypoxic, and this low oxygen tension, from below 1% in the niche itself to 6% in the sinusoidal cavity[216], is involved in the increased proliferation capacity, as well as the maintenance of the naive state and the plasticity of MSCs[209]. In HSCs, the hypoxic environment, which occurs closer to the bone surface and more distanced from the blood capillaries, is associated with a more quiescent state[216]. Figure I-3 represents the BM niche of MSCs and HSCs.

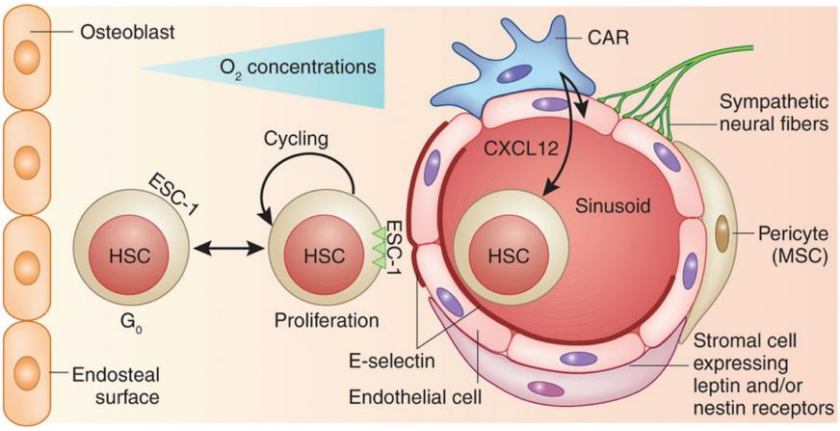


Figure I-3. Perivascular niche of mesenchymal and hematopoietic stem cells[217].

I.4.4.6 Features

One of the features that makes MSCs so attractive in the regeneration field is the immunomodulatory property[218]. Through the secretion of a large spectrum of factors (*i.e.*, paracrine effect; some of the factors involved are presented in Table I-1) or by direct cell-cell contact[219], MSCs interact with the following cells of the immune system:

- T cells – the entrance in the proliferative stage, the S phase, is blocked by inhibition of cyclin D2 and upregulation of p27Kip1; T cells are arrested at the G1 phase; the production of Interferon Gamma (IFN-γ), an inflammatory cytokine, is temporarily reduced[220]; the expression of $Foxp3^+$ regulatory T cells is increased; apoptosis of T cells is activated[218]; the inhibition of the proliferation of T cells is enhanced by hypoxic conditions (1% of oxygen), which occur before the initiation of the inflammatory process[221];
- Monocytes/ Dendritic cells – the hampering of the differentiation of $CD34^+$ and monocytes blocks the generation of dendritic cells (which are initiators of the immune response); dendritic cells have a deficient capacity of activation of T cells[222]; the maturation of dendritic cells is blocked by the inhibition of TNF-α secretion by those cells; the mature dendritic cells are induced to increase the expression of IL-10[223];
- B cells – the proliferation of B cells is blocked by arrest at G0-G1 phases; the differentiation into IgM-, IgG-, and IgA-producing cells is impaired in a dose-dependent manner; the expression of CXCR4, CDCR5, and CCR7 by B cells is downregulated, as is the chemotaxis response[224];
- NK cells – the production of IFN-γ is downregulated in IL-2-stimulated NK cells[223]; the receptors 2B4 (associated with increased cytotoxicity and production of IFN-γ) and NKG2D (involved in cytotoxicity), are downregulated; the proliferation of NK cells is inhibited through the production of TGF-β[225].

A study by Ren and colleagues (2008) showed that, in mice, this immunosuppressive capacity of MSCs is stimulated by pro-inflammatory cytokines like IFN-γ combined with TNF-α, IL-1a or IL-1b[226]. This was also observed by the groups of Renner (2009)[227], Chan (2006), who only reported the importance of IFN-γ in the immune modulatory process[228], English (2007), who only observed a significant role of IFN-γ and TNF-α in the upregulation of Prostaglandin E_2 (PGE_2) expression[229], and Hemeda (2010), who emphasized once again the role of both IFN-γ and TNF-α[230].

The hypoimmunogenicity of MSCs is controversial. In one hand, these cells express MHC class I positive molecules and express null or low levels of MHC class II[231; 232], which would allow them to escape to the deletion mechanisms of NK cells and to the recognition by alloreactive CD4+T cells, respectively[233]. Additionally, MSCs do not express co-stimulatory molecules required to the induction of T cells, such as CD40, CD40L, CD80 and CD86[233]. These facts point to a possible transplantation of MSCs into a Major Histocompatibility Complex (MHC)-incompatible individual without triggering an immune response[233]. On the other hand, NK cells may express a single or certain set of receptors called Killer cell Immunoglobulin-like Receptors (KIR) that in the case of failing the recognition of HLA

class I allele group of MSCs, trigger the alloreactivity of NK cells[229]. This shows that MSCs might escape to the action of some cells from the immune system, but not all.

MSCs also present a homing property, defined as the retention of the cells within the vasculature of a tissue, followed by transmigration across the endothelium[234]. This property is probably resultant from the expression of a set of chemokine receptors, comprising CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6, involved in the homeostatic leukocyte trafficking and cell compartmentalization within the BM and other lymphoid organs[235]. In fact, in the study by Honczarenko and colleagues (2006), the referred set could stimulate the directional migration and *in vitro* chemotaxis of BM MSCs[235]. The treatment of MSCs with a cytokine cocktail containing Flt-3, IL-3, IL-6, Hepatocyte Growth Factor (HGF) and SCF was proved to increase the expression of CXCR4[236]. Consequently, there was a stimulation of the hematopoietic recovery after transplantation into Non-Obese Diabetic (NOD)/SCID mice, associated with an improvement of the short-term homing of MSCs into BM[236], a property that appears to decrease after expansion *in vitro*[237]. MSCs have also been demonstrated to be capable of specific migration into the infarct site, although their delivery through systemic intravenous injection is not very efficient due to the entrapment of the cells in the lungs[238]. In fact, the expansion process induces an increase in their size and the augment of the expression of adhesion molecules, which may contribute to their entrapment in the small capillaries of the lung[238]. The homing of MSCs into the infarcted myocardium may be induced through the localized expression of Stromal Cell-Derived Factor 1 (SDF-1) in the injured site, as shown by the group of Askari (2003)[239], and by natural upregulation of SDF-1, as a response to the infarction, associated with an increased expression of CXCR4 in MSCs, as reported by the group of Cheng (2008)[240]. Other important chemotactic factors for these stem cells are the Platelet-Derived Growth Factor-AB (PDGF-AB), Insulin-like Growth Factor 1 (IGF-1) and the chemokines RANTES, Macrophage-Derived Chemokine and SDF-1, especially after TNF-α stimulation[241].

Other important features of MSCs are the anti-apoptotic, anti-fibrosis and angiogenic properties. MSCs are able to avoid the apoptosis of CMCs by upregulating the cell survival gene Akt after a brief anoxia period[242] and through the secretion of Vascular Endothelial Growth Factor (VEGF) and HGF, which are also angiogenic[243]. In fact, the administration of single angiogenic growth factors is less promising than the use of MSCs for the induction of neovascularization, since the cells are able to adapt their expression profile to hypoxia conditions and throughout time[243]. Moreover, the conditioned medium of MSCs cultured in hypoxic conditions was shown to improve the tube formation by ECs, due to higher concentrations of IL-6 and VEGF, when compared to MSCs from normoxia conditions[244]. For the anti-fibrosis effect, MSCs produce adrenomedullin, which inhibits the proliferation of cardiac fibroblasts and decreases the production of collagen[245] and HGF[246].

The most relevant factors secreted by MSCs and important for the features previously described are present in Table I-1.

Table I-1. Factors secreted by MSCs (adapted from[247]).

Factor	Angiogenic	Anti apoptotic	Anti scarring	Role Chemo attractant	Immuno modulatory	Hematopoietic Supportive
bFGF	✔	✔	✔			
HGF		✔	✔		✔	
IGF-1		✔				
IL-6	✔					✔
LIF					✔	✔
PGE_2					✔	
SCF						✔
SDF-1						✔
TGF-β		✔			✔	
VEGF	✔	✔				
CCL (-2, -3, -4, -5, -7, -10, -20, -26)				✔		
CXCL (-1, -2,- 5, -8, -10, -11, -12)				✔		

I.4.4.7 Clinical Applications

The capacity of MSCs in modulating the immune cell response is extremely beneficial in a different settings, for instances in the context of Graft-Versus-Host-Disease (GVHD)[223]. GVHD is a disease that might follow allogeneic hematopoietic cell transplantation, in which the donor T cells react against the recipient cells, the production of proinflammatory cytokines (like TNF-α) is triggered, and later, the effector T cells start destroying target tissues like gut, liver, skin and lungs[248]. Since MSCs are able to modulate the T cells response[218; 220], downregulate the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ, and increase the production of IL-4[223], these cells, both from BM and AT, may be used to manage the disease[223; 249]. Several clinical trials have already addressed the use of MSCs in the management of GVHD and showed an improvement of the survival rate in patients with a complete response to the treatment[250-252]. Besides preventing or diminishing the severity of this disease, co-transplantation of MSCs with HSCs is a strategy to enhance the engraftment of HSCs[253]. Several other diseases may benefit from the immunomodulatory properties of MSCs, such as experimental auto-immune encephalomyelitis, acute pancreatitis, multiple sclerosis, Crohn's disease, and type 2 diabetes[219].

MSCs might also be used as delivery vectors for pro-apoptotic agents in the treatment of tumours, taking advantage of the chemokine-induced migration, homing and hypoimmunogenic properties[254]. The differentiation potential into osteoblasts and chondrocytes has been shown to be useful in the treatment of fractures, osteonecrosis, spine fusion, cartilage injury, osteoarthritis, and degenerative disc disease, while the paracrine effect of MSCs helps enhancing wound and burns healing, as well as regeneration of spinal cord injuries[255].

Another field in which MSCs present a great potential as a cellular therapy is cardiac regeneration. Since the work of Makino and colleagues, in 1999, about the obtainment of a cardiomyogenic cell line, descendent from mouse BM stromal cells and capable of differentiation into CMCs *in vitro*[206], other studies claimed that BM cells or, more specifically, MSCs, could differentiate into CMCs *in vivo*[121; 256-260] and reduce the fibrotic tissue[259; 260], with no signs of cell fusion[257]. Some of

those studies also reported the angiogenic effect of the transplanted cells[121; 257-259]. The preconditioning of MSCs in hypoxic conditions (0.5% of oxygen, a value close to that found in an ischemic heart) seems to enhance not only their differentiation into CMCs but also their VEGF production and survival rate upon transplantation into a hostile environment with low oxygen tension, high levels of inflammation factors and high concentration of free radicals generated by oxidative stress[261], conditions that might have impaired the successful engraftment of a considerable amount of the transplanted cells in other studies[256]. Similar improvements were observed after preconditioning with SDF-1[262] and a cocktail of growth factors containing basic Fibroblast Growth Factor (bFGF), IGF-1 and bone morphogenetic protein[263].

Korf-Klingebiel and co-workers (2008) showed that, upon transplantation, autologous unselected Bone Marrow Mononucleated Cells (BM-MNCs) were capable of secreting angiogenic factors like angiogenin and VEGF, associated to the healing of the infarct site, and cytoprotective paracrine factors like IGF-1, linked to the rescue of CMCs and to the reduction of the infarct size[264]. Uemura and colleagues also showed that the pre-conditioning of BM-MNCs in anoxia conditions increased the expression of the cell survival gene Akt and, consequently, allowing the prevention for the apoptosis of CMCs was prevented, while the infarct site suffered a size reduction and the left ventricular remodeling was attenuated[242]. Authors also observed a reduced amount of BM-MNCs-derived CMCs, which highlights the importance of the paracrine factors, rather than the cardiomyocyte differentiation, in the cardiac function improvement[242]. The importance of Akt was furthermore explored by the groups of Noiseux (2006) and Gnecchi (2006), who additionally found very low levels of cell fusion[265; 266] and differentiation[265] in the origin of new CMCs. Given that these and other studies pointed out to neovascularization[258; 267-270] and anti-fibrotic effects[259; 260; 271] as important contributors for the improved cardiac function after BM cells transplantation, while true differentiation into CMCs was insignificant[242; 267; 269; 270] or absent[272; 273], it is now accepted that paracrine effects of MSCs play a relevant role in the regeneration of the infarcted heart. Other mechanism by which MSCs might exert their beneficial effects is the stimulation of endogeneous CSCs to migrate to the injured site[274], expand and differentiate into CMCs[275].

Instead of being transplanted, BM-MNCs might also be mobilized into the bloodstream, either by the administration of cytokines[276] or a natural production of these proteins[277], producing similar results to the ones previously described, namely the production of viable myocardium tissue, reduction of the scar size[276; 277], and increase of the EF[276]. Nevertheless, this mobilization process happens within a limited period and might, thus hindering the completion of the cardiac healing process[277].

Despite the claims of their positive impact upon transplantation into an injured myocardium, Breitbach and colleagues showed in 2007 that MSCs or unfractionated BM cells could give rise to calcifications/ossifications when directly injected into the cardiac tissue, which might jeopardize the safety of a cellular therapy based on these cells[278].

I.4.4.7.1 Autologous *vs.* Allogeneic Setting

When performing a cellular therapy, one of the main concerns is the risk of triggering an immune response that may lead to the rejection of the donated cells by the patient[279]. To avoid such

situation, an autologous setting is the choice of election. However, this choice has some disadvantages, namely the harvesting of the cells from the BM, which causes logistic, economic and timing constrains[279], the time consuming expansion of MSCs *in vitro* to achieve a clinically relevant number[196], and the high probability of impairment of the cells when these are extracted from elderly patients who usually have multiple comorbidities[279]. In fact, stromal cells/MSCs from elderly donors have a lower resistance to ischemic environments, are less capable of secreting angiogenic factors[280], as well as having a decreased proliferative and differentiation potential[281], while those from diabetic donors have lower proliferative potential at early passages, decreased survival rate and engraftment *in vivo*[282].

Regarding any other stem cell, MSCs have the advantage of being immunoprivileged[233] and immunomodulatory[218], which allows their transplantation in immunocompatibility-mismatched recipients[279] and the overcoming of the hurdles of autologous MSC. Therefore, allogeneic MSC, extracted from young and healthy donors, might be used to create a high-quality and consistent off-the-shelf product, readily mobilized when needed[196]. The main drawback of this kind of setting is the lack of knowledge regarding the susceptibility of the donor to autoimmune diseases[283].

I.4.5 Clinical Trials and Challenges to Overcome in Cellular Therapy for Acute Myocardial Infarction

On Table I-2, some of the most relevant clinical trials in the context of an AMI or an Ischemic Cardiomyopathy (ICMP) are presented, with data considered important for the comparison between clinical trials, as well as the results that showed a statistically significant improvement.

Table I-2. Clinical trials using stem cells in the context of an AMI or an ICPM. ↓ – Decrease; ↑ – Increase; ADRCs – Adipose Derived Regenerative Cells; Allo – Allogeneic; Auto – Autologous; BM MNCs – Bone Marrow Mononuclear Cells; BMCs – Bone Marrow Cells; c – with control group; CABG – Coronary Artery Bypass Grafting; CAng – Coronary Angiography; CDCs – Cardiosphere-Derived Cells; CPCs – Circulating blood–derived Progenitor Cells; CpSCs – Cardiopoietic Stem Cells; CT – Computed Tomography; db – double blind; DECG – Doppler Electrocardiography; ECG – Electrocardiography; ECHO – Echocardiography; G-CSF – Granulocyte Colony-Stimulating Factor; I – phase I Trial; ICI – Intracoronary Injection; ICMP – Ischemic Cardiomyopathy; II – Phase II Trial; III – Phase III Trial; IMI – Intramyocardial Injection; IV – Phase IV Trial; IVI – Intraventricular Injection; LVAng – Left Ventricular Angiography; LVEF – Left Ventricular Ejection Fraction; LVESV – Left Ventricular End Systolic Volume; LVTM – Left Ventricular Total Mass; mc – multicentre; MI – Myocardial Infarction; MRI – Magnetic Resonance Imaging; nm – not mentioned; PB MNCs – Peripheral Blood Mononuclear Cells; PBSCs – Peripheral Blood Stem Cells; p – placebo controlled; PCI – Percutaneous Coronary Intervention; r – randomized; RAng – Radionuclide Angiography; SE – Spiroergometry; SMSs – Skeletal Myoblast Cells; SPECT – Single-Photon Emission Computed Tomography; TEI – Transendocardial Injection; VG – Ventriculography ; WMSI – Wall Motion Score Index; WT – Wall Thickening.

Trial	Study Design	No. Treated Patients /control	Cell Type	No. of Injected Cells	Delivery Route	Time of Application (days after MI or PCI)	Imaging Method	Follow-up duration (months)	Statistically Significant Results (comparing to control group)
ASTAMI (2006, II)[284]	r, c	50/50	Auto BM-MNCs	6.8×10^7	ICI	6	SPECT, ECG, MRI, CAng	6	No differences
BALANCE (2009)[285]	c	62/62	Auto BMCs	$6.1 \pm 3.9 \times 10^7$	Direct infusion into the infarct-related artery	7 ± 2	ECG, CAng, VG, ECHO	3, 12, 60	↓ Mortality
BONAMI (2011, II)[286]	r, mc	52/49	Auto BM-MNCs	$9.83 \pm 0.87 \times 10^7$	ICI	9.3 ± 1.7	DECG, Rang, SPECT, MRI, CAng	3	No differences
BOOST (2004-2009, I)[287-289]	r, c	30/30	Auto BMCs	$2.46 \pm 0.94 \times 10^9$	ICI	6	MRI, CAng, ECG	6, 18, 60	↑ Global LVEF (6 months); No differences at 18 and 60 months
CADUCEUS (2012-2014, I)[321; 322]	r, c	17/8	Auto CDCs	1.25×10^7 or 2.5×10^7	ICI	45-90	MRI	6, 12	↓ Scar size; ↑ Viable myocardial mass
C-CURE (2013, II and III)[290]	r, mc, c	32/15	Auto CpSCs	7.33×10^8	IVI	192-7 515 (ICMP)	ECHO	6	↑ LVEF; ↓ LVESV; ↑ 6-min walk test
FIRSTLINE-AMI (2005)[291]	r, c	15/15	Auto BM-MNCs	2.8×10^{10}	Mobilization by G-CSF	0-6	CAng, ECHO	4, 6, 12	↑ WT; ↑ LVEF; ↓ WMSI;
FOCUS-CCTRN (2012, II)[292]	r, db, p	61/31	Auto BM-MNCs	1×10^8	TEI	nm (ICMP)	ECHO, SPECT, MRI	6	No differences
HEBE (2011)[293]	r, mc	69/66/65 (BM MNC/ PB MNC/ control)	Auto BM-MNCs and PB-MNCs	$2.96 \pm 1.64 \times 10^8$ BM MNCs or $2.87 \pm 1.37 \times 10^8$ PB MNC	ICI	3-8	MRI	4	No differences

(continuing in the next page)

(continuation of Table I-2)

LateTIME (2011, II)[294]	r, db, p	58/29	Auto BM-MNCs	1.5 x 10^8	ICI	14-21	MRI	6	No differences
Lee *et al.* (2014)[295]	r, c	30/28	Auto MSCs	7.2 ± 0.9 x 10^7	ICI	30	CAng, SPECT, ECHO	6	↑ LVEF
MAGIC (2008, II)[296]	r, mc, db, p	33/34/30 (high dose, low dose, placebo)	Auto SKMs	4 or 8 x 10^8	IMI	nm	ECHO	6	↓ LVESV for high-dose group; 9 cases of arrhythmias in SKM groups
POSEIDON (2012, I and II)[297]	r	15/15 (Allo/ Auto)	Allo and Auto MSCs	0.2, 1 or 2 x 10^8	TEI	nm (ICMP)	ECHO, CT	6, 12, 13	(Relatively to baseline) ↑ 6-min walk test (autologous group); ↓ Infarct size (both groups); ↓ LVESV (allogeneic group); Inverse dose response to cell therapy with regard to EF, LVESV and infarction size improvements
PRECISE (2014, I)[298]	r, db, p	21/6	Auto ADRCs	0.4 x 10^6, 0.8 x 10^6, and 1.2 x 10^6 ADRC/kg	TEI	nm (ICMP)	ECG, ECHO, SPECT, MRI	6, 12, 18	(Relatively to baseline) ↑ LVTM; ↑ WMSI
PROMETHEUS (2014)[299]	r	2/4 (low dose, high dose)	Auto MSCs	2 x 10^7 or 2 x 10^8	IMI	nm (ICMP)	MRI	3, 6, 18	(Relatively to baseline) ↑ LVEF; ↓ Scar mass
REPAIR-AMI (2006-2010, III)[300-303]	r, mc, db, p	101/103	Auto BMCs	2.36 ± 1.74 x 10^8	ICI	3-7	LVAng, MRI	4, 12, 24	↑ LVEF
REVIVAL-2 (2006, IV)[304]	r, db, p	56/58	Auto BMCs	nm	Mobilization by G-CSF	5	SPECT, MRI, LVAng, CAng	4, 6	No differences; 18 cases of restenosis in G-CSF group and 17 in the placebo
SCIPIO (2011, I)[305]	r, c	16/7	Auto CSCs	0.5 or 1 x 10^6	ICI	113 (after CABG) (ICMP)	ECHO, MRI	1, 4, 12	(Relatively to baseline) ↑ LVEF; ↑ WMSI, ↓ Infarct size
STAR (2010)[306]	c	191/200	Auto BMCs	6.6 ± 3.3 x 10^7	Direct infusion into the infarct-related artery	8.5 ± 3.2 years (ICMP)	VG, SE, ECG	3, 12, 60	↓ LVESV; ↑ EF
STEMI (2006)[307]	r, db, p	39/39	Auto BMCs	nm	Mobilization by G-CSF	1-3	MRI, ECHO, CAng	6	No differences
TIME (2012, II)[308]	r, db, p	79/41	Auto BMCs	1.5 x 10^8		3 or 7	MRI	6	No differences
TOPCARE-AMI (2002-2011)[309-311]	r, c	29/30/11 (MNCs/ CPCs/ control)	Auto CPCs or PB-BMCs	1.6 ± 1.2 x 10^7 CPCs or 2.13 ± 0.75 x 10^8 BMCs	ICI	3-7	ECHO, LVAng, MRI	4, 12, 60	(Relatively to baseline) ↓ LVESV; ↑ EF; ↓ Infarct size

From all the clinical trials presented (Table I-2), only the study of Lee and colleagues (2014)[295], POSEIDON[297], and PROMETHEUS[299], used specifically BM-MSCs, showing good results relatively to the baseline or the control group. Nevertheless, the lack of a control or placebo group[297; 299] and technical problems in assessing some parameters[295] were pointed out as a limitation of the studies. The authors of the POSEIDON trial also referred the need for dose-studies due to the unexpected dose response they observed on their clinical trial[297]. In the PROMETHEUS trial, the higher dose of cells led to a more obvious reduction in the segmental scar mass, although the cells were less concentrated[299]. Therefore, the concentration of the cells, instead of the total number of injected cells, might explain the unexpected dose response in the POSEIDON trial[299]. The PRECISE trial used MSCs, but derived from the AT, and showed modest beneficial effects on the cardiac function[298].

The only trial dealing with SKMs presented herein is the MAGIC trial[296]. This study failed in revealing an improvement in the echocardiographic heart function, while showing that these cells were not completely safe since 9 patients suffered from arrhythmias[296].

The clinical trials involving CSCs from either the BM or the heart, namely CADUCEUS, C-CURE and SCIPIO, consistently showed to be safe and to improve the heart condition upon transplantation[290; 305; 312; 313]. However, the administration of the cells was done many days after the MI episode and required a previous expansion *in vitro* to provide a number clinically relevant[312], which is not feasible in the context of an AMI. The mechanism by which they exerted their beneficial action remained to be determined[305; 312], as well as the optimal cell-dose[305].

Much more controversial were the clinical trials using BM cells or (Circulating blood–derived Progenitor Cells) CPCs. Trials where the cells were mobilized through the administration of Granulocyte Colony-Stimulating Factor (G-CSF) showed both good results, in the FIRSTLINE-AMI trial[291], or lack of a significant improvement, in the REVIVAL-2[304] and STEMMI[307] trials. The inefficient homing and engraftment of the cells, possibly caused by the aggressive milieu of the infarcted myocardium, was pointed out as a cause for such efficacy failure[304; 307]. Other constraints further highlighted were the dose of G-CSF used, the timing for treatment, or even the mobilization of cells that are not effective in the improvement of the cardiac function[307].

Almost all the remaining trials failed to reveal a significant difference between the patients treated in the cellular therapy and the control/placebo groups, except for BALANCE[285], REPAIR-AMI[300-303], STAR[306], and TOPCARE-AMI[309-311]. The disparities in the results highlight the need for further work in order to identify the set of patients more prone to benefit from this type of therapy[286; 288; 289; 294; 302], the most appropriate isolation protocol[293], the most effective dose[289], the best delivery strategy for enhancing cells' engraftment[306; 309], the ideal time-frame for cell transplantation[293; 294; 308], and finally the most suitable cell type[294]. Nevertheless, the results of the POSEIDON[297] trial are promising, since allogeneic MSCs could be harvested from healthy donors, expanded until reaching the necessary dose, and administered at any time point, without triggering an immune response and keeping the beneficial potential of MSCs.

II. Aim of the Study and Specific Objectives

The present master thesis aims to comprehensively study the advantages and disadvantages of using MSCs, from autologous or allogeneic origin, in the context of a cell-based therapeutic approach for AMI. The MSCs used were isolated from the BM of AMI patients and healthy donors, as well as from AT samples from healthy donors, aiming towards the identification of both the differences in the performance of healthy and AMI MSCs, and the potential of the AT as a reliable source of MSCs, as alternative to BM.

Studies using BM-derived MSCs (healthy and AMI donors) and ADSCs from healthy donors were performed under normoxic and hypoxic conditions, to ascertain the impact of a low oxygen tension, which exists both in the BM and the infarct site, on the performance of MSCs, namely in terms of *ex vivo* proliferative capacity. On the other hand, an angiogenic and tube formation assay was established to attest the ability of these cells in forming or integrating micro blood vessels, which are important for the revascularization process of the infarcted myocardium. An oxidative stress resistance quantification assay was also established to study the resistance of the MSCs to the presence of oxidative species released to the tissue as a consequence of the ischemic episode. Finally, the secretome of MSCs, both from healthy and AMI donors, was studied.

Regarding the AT as a viable source of MSCs for cell therapy settings, including AMI, two different isolation methods for the SVF were compared, the conventional one based on an enzymatic processing, and a method based on mechanical forces. The objective was to explore an isolation method free of undefined and animal derived components, which can potentially be more cost-effective, and closer to a clinical grade product, comparing its yielding to the conventional isolation method.

This master thesis was conducted at the Stem Cell Bioengineering and Regenerative Medicine Laboratory, located at the Instituto Superior Técnico Tagus Park *campus*, in collaboration with Hospital de Santa Marta (Centro Hospitalar Lisboa Central), and Clínica de Todos-Os-Santos (Lisboa).

III. Materials and Methods

III.1 Human Samples

The BM samples from healthy donors and AMI patients were obtained from Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, and Hospital de Santa Marta, EPE, Lisboa, Portugal, respectively. These samples were previously processed and the MSCs isolated were cryopreserved and stored at SCBL-RM. ADSCs were obtained from Pennington Biomedical Research Center Institutional Review Board, USA and kept cryopreserved at SCBL-RM. The AT samples that were processed in the scope of this work were obtained from Clínica de Todos-Os-Santos, Lisboa, Portugal. All the samples were obtained after informed consent of the patients and their harvesting and collection was performed in accordance with the protocols of the respective institution. The Human Umbilical Vein Endothelial Cells (HUVEC) used in the present work were a HUVEC cell line from BD Biosciences.

III.2 Cell Cryopreservation

To cryopreserve cells, one of the following media was used: Recovery™ - Cell Culture Freezing Medium (Gibco®) or FBS containing 10% of Dimethyl Sulfoxide (DMSO, Sigma). Both media have DMSO, a cryoprotectant which avoids the rupture of cells by the formation of ice crystals[314].

The cells were centrifuged at 1250 rpm for 7 minutes at room temperature and re-suspended in one of the media mentioned above. The total volume was quickly distributed through 1 mL cryovials (Thermo Scientific), which were then stored in a -80°C freezer and later on transferred to liquid nitrogen for a long-term storage. When cryopreserving MSCs, ADSCs or HUVEC, a density of around 0.5×10^6 to 1×10^6 cells/vial was used. In the case of cryopreserving SVF, the density varied from 1×10^6 to 4×10^7 cells/vial.

III.3 Cell Thawing

Cells were thawed by immersion in a 37°C water bath, for 1-2 minutes, and immediately re-suspended in IMDM supplemented with 20% FBS and 1% AA, in a proportion of 1 to 5, regarding the volume of the vial. The cells were then centrifuged at 1250 rpm for 7 minutes at room temperature and re-suspended in the appropriated medium.

III.4 Evaluation of the *in vitro* Proliferative Potential of Mesenchymal Stem/Stromal Cells

Cells from both adipose and BM origin were expanded on T-25 flasks (BD Falcon™), incubated at 37°C, 5% CO_2, and one of two conditions, normoxic or hypoxic, with 20% or 2% O_2, respectively. Cells were plated at an initial density of 3 000 cells/cm^2 and the medium was changed every 3 to 4 days. The medium used was DMEM+10% MSC FBS. When reaching 70 to 80% confluence, the detachment procedure was performed. Briefly, cells were washed with PBS and harvested with Accutase (Sigma) for 5-7 minutes at 37°C. IMDM+10% FBS was added, in a proportion of 1:2, to inactivate the enzyme, and the cells were centrifuged at 1250 rpm for 7 minutes. After discarding the

supernatant and re-suspending the pellet in the culture medium, the number of cells and their viability was accessed using the Trypan Blue dye (Gibco®) exclusion method and a Neubauer chamber under an optical microscope. The cells were re-plated into new culture flasks at the same initial conditions.

The proliferation study was performed from passage number 3 (P3) until P9, and at every passage different parameters were determined according to the following equations, namely:

- Cell density – ratio between the number of viable cells at the end of the passage and the area in which the cells were plated;
- Fold Increase (FI) – ratio between the number of viable cells at the end of the passage and the number of viable cells plated at the beginning of the same passage (Eq. III-1);
- Cumulative Fold Increase (CFI) – the product of the FI of each consecutive passage until the present passage (Eq. III-2);
- Population Doublings (PD) – ratio between the base ten logarithm of the FI of the passage and the base ten logarithm of 2, corresponding to the number of doublings the population underwent on that passage (Eq. III-3);
- Cumulative Population Doublings (CPD) – sum of the PD of each consecutive passage until the present passage (Eq. III-4).

$$Fold\ increase = \frac{number\ of\ viable\ cells\ at\ the\ end\ of\ the\ passage\ n}{number\ of\ viable\ cells\ initially\ plated\ at\ the\ passage\ n} \quad \textbf{(Eq. III-1)}$$

$$Cumulative\ fold\ increase = \prod_{i=1}^{n} fold\ increase\ of\ passage\ i \quad \textbf{(Eq. III-2)}$$

$$Population\ doublings = \frac{\log_{10} fold\ increase\ in\ passage\ n}{\log_{10} 2} \quad \textbf{(Eq. III-3)}$$

$$Cumulative\ population\ doublings = \sum_{i=1}^{n} population\ doubling\ of\ passage\ i \quad \textbf{(Eq. III-4)}$$

Other parameter that was considered was the time the cells took to expand from P3 to P9.

III.5 Mesenchymal Stem/Stromal Cells Secretory Potency Assays

Enzyme-Linked Immunosorbent Assay (ELISA) kits were performed to compare the production potency of a specific group of growth factors between cells from both adipose and BM origin and expanded under hypoxic or normoxic conditions. These kits allow the detection of diminutive quantities of proteins and other antigens through their binding to a specific antibody[315]. The antibodies are then detected by a secondary enzyme-coupled antibody which uses a chromogenic substrate to produce a colour or fluorescence change[315]. The quantitative or qualitative measurements of such event allow the inference of the amount of antigen present in the sample[315].

The growth factors studied using ELISA kits (RayBio®) were IL-6, SDF-1, TGF-β, and VEGF, due to their importance in the cardiac repair by stimulating angiogenesis[268], mobilizing BM progenitor cells[316], amongst others. To evaluate the effect of inflammatory cytokines, released by damaged tissues, in the production of the cytokines mentioned above, the cells were also stimulated with TNF-α and IFN-γ, which are proven to induce an increased expression of cytokines, such as IL-6[317] and TGF-β[318].

At the end of P3 or P4, the cells were detached according to the protocol previously described in section III.4 and plated into 4 wells of a 24 well plate, at a density of 3 000 cells/cm^2, using DMEM+10% MSC FBS as medium. After 4 days in culture at 37°C, 5% CO_2 and normoxic or hypoxic conditions, the medium was replaced by fresh DMEM+10% MSC FBS, being also supplemented with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α in 2 wells, which constituted the inducted wells. The non-treated cells were considered the control wells. Cells were further cultured for 4 days, after which the culture medium was collected, centrifuged at 1500 rpm for 10 minutes at room temperature, and stored at -80°C. The determination of cell number and viability for both the stimulated and control wells was done following the detachment procedure previously described in section III.4.

All the reagents for each kit were prepared according to their respective instructions before starting the assay. The remaining protocol was identical amongst the kits.

After allowing the reagents and samples to reach room temperature, 100 µL of each standard and sample were added to pre-determined wells of the microplate, which was incubated for 2.5 hours at room temperature with gentle shaking. By the end of the incubation, the solution was discarded, followed by a washing step with 300 µL of 1x Wash Solution, repeated 4 times. After the last wash, the microplate was inverted and blotted against a clean paper towel to remove any remaining of the Wash Solution. Afterwards, 100 µL of 1x prepared biotinylated antibody were added to each well and incubated for 1 hour at room temperature with gentle shaking. After discarding the solution, the microplate was once again washed 4 times with the Wash Solution, followed by the addition of 100 µL of prepared Streptavidin solution to each well and incubation for 45 minutes at room temperature with gentle shaking. This solution was also discarded and the washing procedure repeated as described above. The final incubation occurred with 100 µL of TMB One-Step Substrate Reagent for 30 minutes at room temperature in the dark with gentle shaking. When the incubation ended, 50 µL of Stop Solution were added to each well and the absorbance of the microplate was read at 450 nm using a reader (Infinite® 200 PRO, Tecan) and the software *Tecan i-control 1.8*.

The absorbance values of the standard wells were used to produce a standard curve, which was then used to estimate the concentration of the respective growth factor in each sample.

III.6 *In vitro* Angiogenic Assay and Tube Incorporation Assay

The *in vitro* angiogenic potential of MSCs, from both adipose and BM origin and expanded under hypoxic or normoxic conditions, was accessed through three assays: (i) an angiogenic assay, where MSCs were seeded alone and compared to HUVEC, (ii) a tube incorporation assay, where MSCs were seeded together with HUVEC, and (iii) an angiogenic assay with conditioned medium, where the culture medium of MSCs was used to culture HUVEC. All the MSCs used were from P3-P4. The HUVEC cells were expanded on T-75 flasks from P5-P8 and incubated at 37°C, 5% CO_2. These cells were plated at an initial density of 3 000 cells/cm^2 and the medium was changed every 3 to 4 days. The medium used was Endothelial Growth Medium (EGM)-2 (Lonza). When reaching 70 to 80% confluence, the detachment procedure was performed as described in section III.4.

In all the assays, wells from a 96 well black/clear tissue culture treated plate flat bottom with lid (BD Falcon™) were coated with Matrigel (10 mg/mL, 0.4 mL, BD Biosciences), which was allowed to

polymerize overnight at 37°C. Each sample was then placed in triplicate wells, using a total volume of 50 µL/well of cells suspended in EGM-2, except for the conditioned medium assay.

In the angiogenic assay, 10 000 cells of HUVEC, MSCs expanded in normoxia and MSCs expanded in hypoxia were seeded per each well. In the angiogenic assay with conditioned medium, 20 000 cells of HUVEC in EGM-2 medium, HUVEC in normoxia conditioned medium and HUVEC in hypoxia conditioned medium were seeded per each well. The conditioned media of the cells were collected on the day of the angiogenic assay and corresponded to media of cells cultured for 1-4 days. When the collection was not possible, and if available, the stored media from the control cells obtained as described in section III.5 were used.

In the tube incorporation assay, MSCs were previously labelled with 10 µg/mL DiI-Acetylated-Low-Density Lipoprotein (DiI-Ac-LDL, Biomedical Technologies Inc.) in EGM-2 media at 37°C for 20 minutes. After the incubation, the cells were washed by adding 2 mL of PBS and centrifuging at 1250 rpm for 7 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in EGM-2 media. In this case, 20 000 HUVEC were seeded in each well, three to form the control group, and in six to form the co-culture groups. From these, three were posteriorly seeded with 5 000 MSCs expanded in normoxia per well, and the remaining were seeded with 5 000 MSCs expanded in hypoxia per well.

After seeding, the cells were incubated for approximately 8 hours at 37°C and 5% CO_2, in normoxia conditions. Afterwards, each well was washed with 200 µL of PBS. The cells were then labelled with 100 µl/well of 8 µg/mL of calcein (BD Biosciences) fluorescent dye in PBS and incubated at 37°C and 5% CO_2 for 30 minutes. Each well was again washed with 200 µL of PBS and the tube-like structures were observed under a fluorescence microscope (Leica) and using the software *Nikon ACT-1 2.70*, which was also used to acquire images of each well. The software *ImageJ 1.49b* was used to determine the tube length, the number of tubes and the total number of connections.

III.7 Oxidative Stress Resistance Quantification Assay

To access the resistance of MSCs, from AT or BM origin and expanded under hypoxic or normoxic conditions, to the presence of Hydrogen Peroxide (H_2O_2), an oxidative stress resistance quantification assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) and the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen™), following the instructions of the kits. The first kit was used to measure, in a quantitatively way, the Lactate Dehydrogenase (LDH) released to the medium as a result of cell lysis, in order to estimate the cytotoxicity promoted by the presence of H_2O_2. The aim of using the second kit was to determine the percentage of early apoptotic cells and late apoptotic cells.

At the end of P3 or P4, the cells were detached according to the protocol previously described in section III.4 and plated into 5 wells of a 12 well plate, at a density of 3 000 cells/cm^2 or 6 000 cells/cm^2, using DMEM+10% MSC FBS as medium. After 1 or 4 days in culture at 37°C, 5% CO_2 and normoxic or hypoxic conditions, the medium was replaced by fresh DMEM+10% MSC FBS or MEM Alpha Medium (α-MEM, Gibco®) supplemented with 10 % or 5% MSC FBS and 1% of AA, a medium without phenol red, used to reduce the background absorbance in the CytoTox 96® Assay. 100 or 200

μM of H_2O_2 were added to 2 of the seeded wells and the plate was incubated for 2, 6, 24 or 48 hours at 37°C, 5% CO_2 and normoxic conditions, regardless the original conditions of the previous incubation. The CytoTox 96® Assay and the FITC Annexin V staining procedure were then performed simultaneously as described ahead (sections III.7.1 and III.7.2).

III.7.1 CytoTox 96® Non-Radioactive Cytotoxicity Assay

For the CytoTox 96® Assay, after the incubation period, 1 of the 3 wells free of H_2O_2 received 15 μL of Lysis 10X Solution (9% (v/v) Triton® X-100 in water) per 200 μL of culture medium, and the plate was incubated for 45 minutes at 37°C, 5% CO_2 and normoxic conditions. After this period, the supernatant of each well was collected, centrifuged at 1500 rpm for 10 minutes at room temperature, and 50 μL/well of each sample were transferred to 3 wells in a 96 well plate, in order to have triplicate results. 50 μL/well of reconstituted Substrate Mix were added to each well and the plate was incubated for 30 minutes at room temperature, protected from the light. During this period, an enzymatic reaction involving the conversion of 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phe-nyltetrazolium chloride (INT) into a formazan product was catalyzed by the LDH in suspension and the diaphorase present in the substrate mixture[319]. The enzymatic reaction was then stop by the addition of 50 μL/well of Stop Solution and the absorbance of the wells was read at 490 nm using a reader (Infinite® 200 PRO, Tecan) and the software *Tecan i-control 1.8*.

The percentage of cytotoxicity for each sample was calculated as the ratio between the corrected and normalized absorbance of the wells that received H_2O_2 and the corrected and normalized absorbance of the well that received the lysis solution, which corresponds to the maximum value of cytotoxicity, as presented in the (Eq. III-5). The correction of the referred values was done by subtracting the absorbance value of the wells that had only DMEM or α-MEM medium, while the normalization was performed by dividing the corrected value by the number of dead cells in the correspondent wells, estimated during the FITC Annexin V staining procedure (section III.7.2).

$$\%\ cytotoxicity = \frac{\frac{absorbance_{H_2O_2} - absorbance_{medium\ only}}{number\ of\ dead\ cells_{H_2O_2}}}{\frac{absorbance_{lysis\ solution} - absorbance_{medium\ only}}{number\ of\ dead\ cells_{lysis\ solution}}} \times 100 \qquad \textbf{(Eq. III-5)}$$

III.7.2 FITC Annexin V Staining

For the FITC Annexin V staining, the cells from all the wells, except the one that received lysis solution, were detached through the procedure previously described in section III.4. After counting both alive and dead cells using the Trypan Blue dye exclusion method, the cells were centrifuged at 1250 rpm for 7 minutes at room temperature and re-suspended in 1X Binding Buffer. Each sample was then distributed by 2 FACS tubes (100 μL of cell suspension/tube), one of them being the control, with no staining, and the other receiving 2.5 μL of FITC Annexin V and 5 μL of Propidium Iodide (PI). While Annexin V binds to phosphatidylserine residues that redistribute from the inner to the outer leaflet of the cell membrane during the early stage of apoptosis, PI intercalates into DNA after entering into the cell when the membrane integrity is lost[320]. The stained tubes were gently vortex and incubated for 15 minutes at room temperature, protected from the light. After the incubation, 200 μL of

1X Binding Buffer were added to every tube and the samples were analysed by flow cytometry within 1 hour.

The software *FlowJo vX.0.7* was used to determine the percentage of early apoptotic cells, being positive for Annexin V and negative for PI, and the percentage of late apoptotic cells, which are positive for both Annexin V and PI. The values were corrected by subtracting the correspondent percentages of the respective control samples. The estimation of the number of cells that died during the incubation with H_2O_2 was made by subtracting the number of viable cells of the wells that received the H_2O_2 treatment to the number of viable cells of the control wells (without H_2O_2 treatment). The number of cells that died during the incubation with lysis solution was considered to be half the viable cells counted in the control group (since the lysis group had only half the cells before the treatment).

III.8 Stromal Vascular Fraction and Adipose-derived Stem/Stromal Cells Isolation

The AT samples were processed at the SCBL-RM, Instituto Superior Técnico, and the SVF was isolated using two different methods, adapted from the literature[321].

III.8.1 Enzymatic Method

Briefly, 50 to 200 mL of aspirate and an equal volume of pre-warmed Phosphate Buffered Saline solution (PBS, Gibco®) containing 1% of Antibiotic-Antimycotic (AA, Gibco®) were mixed in a 500 mL bottle. The sample was washed by gentle shaking of the bottle. After resting, two phases appeared, an AT supernatant and an aqueous infra-natant. The latter was discarded and the tissue was further washed 2-3 times until the tissue became more yellow indicating the removal of erythrocytes. The resultant volume was distributed into 50 mL tubes and mixed with an equal volume of 0.1% collagenase II solution in Hank's Buffered Salt Solution (HBSS, both from Gibco®) previously filtered and pre-warmed in a 37°C bath. The tubes were incubated at 37°C for 30 minutes in a thermomixer (Eppendorf). The digested product was subjected to a vacuum filtration using a Steriflip filter unit (Millipore) and centrifuged at 1250 rotations per minute (rpm) for 7 minutes at room temperature. The topmost layers of oil, fat and collagenase solution were discarded and the SVF pellet was washed by re-suspension in 10 mL of Iscove's Modified Dulbecco's Medium (IMDM, Gibco®) supplemented with 10% Fetal Bovine Serum (FBS, Gibco®) and 1% AA (IMDM+10% FBS). This suspension was once more centrifuged at 1250 rpm for 7 minutes at room temperature and the pellet was re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®) supplemented with 10% MSC qualified Fetal Bovine Serum (Hyclone®) and 1% AA (DMEM+10% FBS). The number of cells and their viability was accessed using Turk's Reagent Solution (Merck Millipore) dye exclusion method and a Neubauer chamber under an optical microscope (Olympus).

III.8.2 Non-Enzymatic Method

Briefly, 25 to 200 mL of aspirate and 50 mL of PBS containing 1% of AA were poured into a 250 mL bottle. The bottle was vigorously shaken by hand for 1-2 minutes and after the separation of both phases, the aqueous infra-natant was saved into 50 mL Falcon tubes. This wash-step was repeated 2-

3 times. The recovered infra-natant was centrifuged at 1250 rpm for 7 minutes at room temperature and the SVF pellet was washed by re-suspension in 10 mL of IMDM+10% FBS. This suspension was again centrifuged at 1250 rpm for 7 minutes at room temperature and the pellet was re-suspended in DMEM+10% MSC FBS. The number of cells and their viability was accessed using the same method described in section III.8.1.

For both methods, after the determination of the number of cells isolated, part of the SVF was cryopreserved, according to the procedure described further on, and the remaining cells were plated into T-75 or T-175 (BD Falcon™) flasks at a high initial density of 100 000 cells/cm^2 in order to isolate ADSCs, based on the adherence to the plastic surface.

For plating, cells were re-suspended in DMEM+10% MSC FBS or in StemPro® MSC SFM (Gibco®), a Xeno(geneic), Serum-Free (XSF) medium, and cultured at 37°C with 5% CO_2. After 24 hours the medium was exchanged to remove non-adherent cells contaminants. Subsequently, the medium was changed every 3 to 4 days. When reaching 70-80% confluence, cells were detached, according to the procedure described in section III.4, and re-plated at the same initial conditions, except for the initial cell density, which decreased to 3 000 cells/cm^2. When using the XSF medium, the flaks were previously coated with CELLstart™ (Gibco®) for 1 hour at 37°C, diluted in a proportion of 1:100, in order to create an attachment substrate.

III.9 Adipose-derived Stem/Stromal Cells Characterization

The ADSCs isolated using the methods described in section III.8 were further characterized based on immunophenotypic analysis.

III.9.1 Immunophenotype analysis

ADSCs from passages P0 to P3 were analyzed by flow cytometry using a panel of mouse anti-human monoclonal antibodies, Phycoerythrin (PE), Fluorescein Isothiocyanate (FITC) and Alexa 488 conjugated, against the cell surface proteins listed in Table VII-2 from Annexes. PE is an orange protein with an emission peak at 578 nm, FITC and Alexa 488 conjugated are a green fluorochrome with an emission peak at 519 nm[322]. These fluorochromes can be excited by a 488 nm blue laser[322].

Briefly, the cells were centrifuged at 1250 rpm for 7 minutes at room temperature, re-suspended in PBS and distributed by FACS tubes (100 µL per tube). After, 5-10 µL of the respective antibody were added to each FACS tube, which were then incubated at room temperature for 15 minutes, in the dark. The removal of the excess of non-labelling antibodies was performed by adding 2 mL of PBS to each tube, followed by a centrifugation at 1000 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended and fixed in 1% Paraformaldehyde (PFA, Sigma®). If the analysis was not performed immediately, the cells were stored at 4°C. Finally, each sample was analysed by flow cytometry (FACSCalibur equipment, Becton Dickinson) through the acquisition of 10 000 events using the data acquisition software *CellQuest* (Becton Dickinson). The results were analysed through *Flowing Software 2.5.1*.

III.10 Statistical Analysis

Throughout this thesis, the results concerning more than one donor are presented as mean ± Standard Error of the Mean (SEM), an inferential statistic that gives information on the precision of the sample value concerning the true value of the population[323].

Student's unpaired t-test was performed to analyse two sets of data. One-way ANOVA with Tukey *post hoc* test for correction of multiple comparisons was performed to analyse three sets of data. Two-way ANOVA with Bonferroni *post hoc* test or with Tukey *post hoc* test for correction of multiple comparisons was performed to analyse grouped sets of data. A *p* value inferior to 0.05 was considered as statistically significant. The statistical analysis was done through the software *GraphPad Prism 6.05*.

IV. Results and Discussion

IV.1 Donor Samples

Different human MSCs donors were used in this work. Each donor was identified with a specific code: for BM donors the code enclosed information about gender and year of birth of the donor, year of harvesting and the origin of the cells, and for AT donors the code comprised information about the day, month and year of sample processing or harvesting (the latter was chosen when two samples were processed at the same day). All the donors studied in the present work are shown in Table VII-3 from Annexes.

The information about the age and gender of the donor, as well as the source of the sample is important since these factors affect the performance of the cells and the yield of MSCs obtained after processing. For example, for donors over 60 years and donors aged 25-50, BM-MSCs stop proliferating around P7 or P15, respectively[324]. Beyond the hampering of the proliferation, which is also observed in MSCs from AT[325], age also impairs the osteogenic differentiation[324; 325] and the resistance to oxidative stress[325], induces changes in the phenotype of the cells[326] and increases the apoptosis rate[325; 326]. With an increasing age, the number of MSCs present in the BM also declines[193]. Female gender is associated to a significant different expression of some surface markers, smaller BM-MSCs, higher presence of colony forming cells, and increased immunosuppression of T cells[327]. The source of MSCs has likewise an impact in their features, namely in the proliferation, differentiation potential, angiogenic cytokines expression, and resistance to oxidative stress[193]. Due to their hypoimmunogenic[233] and immunomodulatory[218] properties, MSCs are suitable for the creation of a high-quality and effective off-the-shelf product, based on allogeneic cells extracted from young and healthy donors[283], thus avoiding some of the mentioned factors that might impair their performance.

The BM donors studied in this work were all of male gender and had an average age of 51 ± 8 years and 49 ± 4 years, for healthy and AMI donors, respectively. The MSCs from healthy donors might be considered to be at an intermediary stage, between adult (25-50 years) and old (over 60 years), thus, their proliferative potential and oxidative stress resistance may be negatively affected by age[326].

Regarding the AT samples that were processed, 9 out of 11 samples were from female donors, which corresponds to a percentage of 82% and 18% for female and male donors, respectively. The average age of these donors was 41 ± 5 year. Consequently, the derived MSCs might be classified as adult, according to Choudhery and co-workers (2014), and their properties are not expected to be much altered when compared to young cells (from donors younger than 30 years)[325].

IV.2 Comparison of Mesenchymal Stem/Stromal Cells from Adipose Tissue and Bone Marrow (Healthy and Acute Myocardial Infarction Patients)

IV.2.1 Proliferative Potential Evaluation

The MSCs from either AT or BM (from healthy or AMI patients) were expanded from P3 to P9 and, at each passage, the following parameters were estimated (as described in section III.4): (1) cell density, (2) FI, (3) CFI, (4) PD, and (5) CPD. The time that the cells took to expand from P3 to P9 was

also registered. Such parameters are important in order to determine how the proliferative potential is affected by the source of the MSCs, the oxygen tension during the cell culture (normoxia or hypoxia), and the health state of the donors. The results for the mentioned parameters are displayed at Table IV-1 and Figure IV-1. The oxygen tension of 2% was chosen to simulate hypoxic conditions because it is a value in the range that is supposed to exist in the BM (from below 1% until 6%)[216] and, according to dos Santos and colleagues 2010[328], 2% O_2 induced significant differences in the cell growth of BM-MSCs when compared to normoxic conditions (20% O_2), which was not verified with 5% O_2[328].

Table IV-1. Time of expansion (in days) from P3 to P9, of healthy BM-MSCs, ADSCs, and AMI BM-MSCs, under hypoxic (2% O_2) or normoxic (20% O_2) conditions. For both the oxygen tensions, n = 3 for every type of cell. * p < 0.05 between Healthy BM MSCs (Normoxia) and AMI BM MSCs (Hypoxia) and between Healthy BM MSCs (Hypoxia) and AMI BM MSCs (Hypoxia).

		Time of Expansion from P3-P9 (days) (mean ± SEM)		
		Healthy BM MSC	**ADSC**	**AMI BM MSC**
Oxygen Tension	**Normoxia (20% O_2)**	31 ± 0.67	33± 2.60	36 ± 4.84
	Hypoxia (2% O_2)	31 ± 0.33	34 ± 0.88	38 ± 4.18*

Table IV-1 shows that the time to expand cells from P3 to P9 was not very different amongst the two oxygen tensions explored. This parameter was also very similar for healthy BM-MSCs and ADSCs, but registered a slight increase for AMI BM-MSCs. Although such variation was not significant for the normoxia condition, a statistically significant difference was obtained between AMI BM-MSCs expanded in hypoxia and healthy BM-MSCs expanded in both normoxia and hypoxia conditions, with the p value being 0.0391 in both situations.

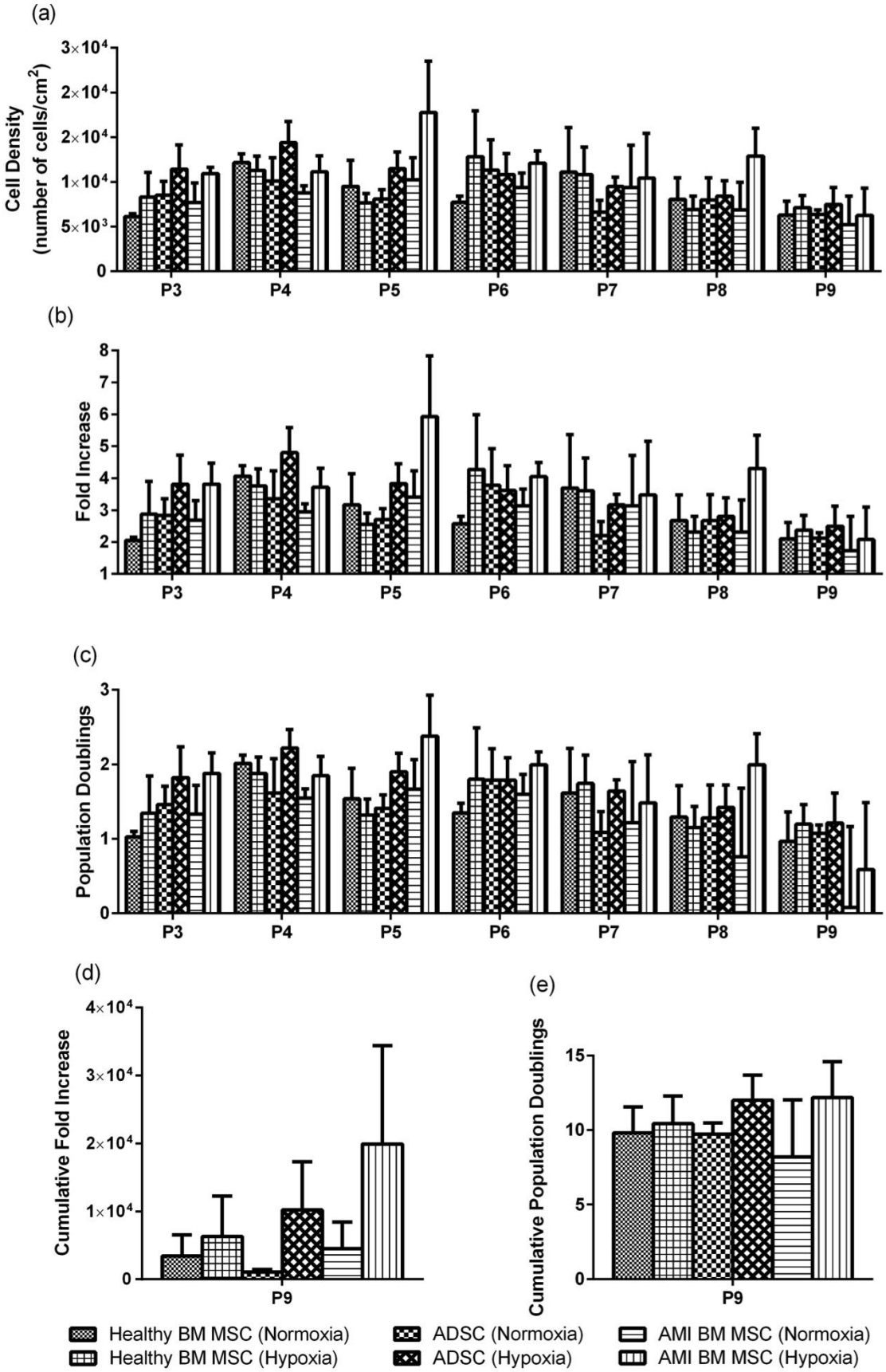


Figure IV-1. Evaluation of the proliferative potential of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions. (a) Cell Density (number of cells/cm^2); (b) Fold Increase; (c) Population Doublings; (d) Cumulative Fold Increase (e) Cumulative Population Doublings. In all the graphics, n = 3 for every type of cell at every passage. Results are presented as mean ± SEM.

Looking at the evolution of cell density (Figure IV-1 a), FI (Figure IV-1 b), and PD (Figure IV-1 c) with the passages, a general trend of increasing values is observed approximately until half the proliferation study. In the three graphics, peak values were obtained for healthy BM-MSCs at P4 and P6, for ADSCs at P6 and P4, and for AMI BM-MSCs at P5 and P5, for normoxic and hypoxic conditions respectively. The only exception was the peak value for the PD of healthy BM-MSCs expanded in hypoxia, which occurred at P4 and not at P6, despite the reduced differences between the values, 1.9 ± 0.2 and 1.8 ± 0.7, respectively. After the peaks, there was an overall tendency of a successive decreasing in values until P9. It is noteworthy the fact that hypoxia conditions induced higher cell densities throughout the passages, but not for healthy BM-MSCs at P4, P5, P7 and P8, and ADSCs at P6. At P7, the trend was normalized for the PD of the healthy BM-MSCs, while for the same parameter, ADSCs displayed the same PD at P6. None of the differences found were statistically significant.

AMI BM-MSCs attained both the smallest (in normoxic conditions) and the highest (in hypoxic conditions) values for the peaks in the referred parameters. For cell density, the values reached were $1.0 \pm 0.3 \times 10^4$ cells/cm^2 and $1.8 \pm 0.6 \times 10^4$ cells/cm^2, while for FI the values obtained were 3.4 ± 0.8 and 5.9 ± 1.9, and for PD, 1.7 ± 0.4 and 2.4 ± 0.6.

Other marks of this proliferative study may be highlighted. For ADSCs and AMI BM-MSCs, the last passage attained the lowest FI of the proliferative study, while for healthy BM-MSCs, the lowest value was obtained at P3 in normoxia (although it was very similar to the one at P9, 2.1 ± 0.1 versus 2.1 ± 0.5 respectively) and at P8 in hypoxia (even though it was close to the one at P9, 2.3 ± 0.5 versus 2.4 ± 0.5). AMI BM-MSCs expanded in normoxia were the cells with the lowest FI of the all study, only 1.7 ± 1.1. The lowest PD were also achieved at the highest passage, except for healthy BM-MSCs expanded in hypoxia, in which such minimum was obtained at P8 with a value of 1.2 ± 0.29, against 1.2 ± 0.26 at P9. The values for the minimum PD ranged from 0.1 ± 1.1 for AMI BM-MSCs expanded in normoxia, to 1.2 ± 0.4 for ADSCs expanded in hypoxia.

The CFI (Figure IV-1 d) was crescent throughout the passages. In both the CFI and CPD graphics (Figure IV-1 d and e), the results for the cells expanded in hypoxia conditions are always superior to those of cells expanded in normoxia, even if only slightly.

At the final passage, the pairs of CFI values (normoxia and hypoxia) for healthy BM-MSCs, ADSCs, and AMI BM-MSCs, were $3.4 \pm 3.1 \times 10^3$ and $6.3 \pm 5.9 \times 10^3$, $1.1 \pm 0.4 \times 10^3$ and $1.0 \pm 0.7 \times 10^4$, $4.5 \pm 3.9 \times 10^3$ and $2.0 \pm 1.4 \times 10^4$, respectively. In this graphic it is clear that healthy BM-MSCs expanded in both oxygen tensions had a higher proliferative capacity than ADSCs expanded in normoxia, though the latter cells, expanded in hypoxia, were capable of overcoming the performance of the referred cells. Although both healthy BM-MSCs and ADSCs from hypoxic conditions had an enhanced performance relatively to AMI BM-MSCs expanded in normoxia, the cells with the greatest proliferative potential were AMI BM-MSCs expanded in hypoxia, although with no statistical significance.

The ratio of the CPD between hypoxia and normoxia was 1.1, 1.2, and 1.5 for healthy BM-MSCs, ADSCs, and AMI BM-MSCs, respectively. The higher ratio was obtained for AMI BM-MSCs

since these cells displayed the lowest (8 ± 3.8) and highest (12 ± 2.4) CPD at P9 for normoxia and hypoxia conditions, respectively.

During the year of 2006, Ren and colleagues analysed the effect of a hypoxic expansion (8% O_2) versus a normoxic expansion (21% O_2) in mouse BM-MSCs[329]. After 8 days in culture, there was a 2.8-fold increase in cell number under hypoxic conditions, compared to normoxic[329]. In the present work, 8 days corresponded approximately to P5 and, for this passage, the ratio of cell densities between hypoxic expansion and normoxic expansion was 0.8 and 1.7 for BM-MSCs from healthy and AMI patients, respectively. As previously highlighted, healthy BM-MSCs did not follow the tendency of having superior results for hypoxia conditions at all the passages in non-cumulative parameters, although the differences were not significant and could be the result of variability introduced by the operator in this study. Beyond this, the oxygen tensions used herein and in the referred study were not the same, nor the species from which the cells were harvested[329], which might have contributed to the differences observed in the values of the ratios. Nevertheless, it is noteworthy that for the AMI-MSCs, the fold-increase in cell number also favoured the hypoxic conditions, as seen by Ren and colleagues (2006)[329]. Despite the punctual discordance between the non-cumulative values obtained and the expectations, it is noteworthy that the cumulative parameters supported the higher expansion capacity of any cell type under hypoxic conditions. The authors associated this improved potential to an augmented number of MSCs entering cell cycle, namely at phases G2-M/S, induced by the hypoxic conditions[329].

In 2010, dos Santos and colleagues compared the cell growth of BM-MSCs under normoxia (20% O_2) and hypoxia (2% O_2) and verified that after 3 passages (correspondent to 12 days in culture), the CFI was significantly different amongst the two conditions, 30 ± 6 and 40 ± 10, respectively[328]. In the present work, 12 days of cell culture occur between P5 and P6. For those passages, the values of the CFI in normoxia and hypoxia were 25 ± 6.2 and 26 ± 8.1, 68 ± 22.5 and 129 ± 85.0, respectively. There is, therefore, an agreement between the values obtained herein and those reported in the literature[328], with hypoxia inducing higher CFI than normoxia conditions.

The study by Basciano and co-workers (2011) further supported the proliferation enhancement of BM-MSCs after P2 upon exposure to hypoxia conditions (5% O_2), compared to normoxia conditions (21% O_2)[330]. This low oxygen tension, hypothesized to mimic the niche conditions in the BM, also stimulated the cells to express more ECM and adhesion molecules like CXCR4[330], with this latter having potential to enhance the engraftment of MSCs to infarcted myocardium[240]. Berniakovich and Giorgio (2013) also reported the existence of an initial lag phase in the proliferation of BM-MSCs, induced by hypoxia (3% O_2), that preceded a superior proliferative stage starting at day 7, caused by a higher number of cells in S-G2/M phase[331].

The positive effect of low oxygen tensions observed herein for ADSCs was also seen by Estrada and colleagues (2012)[332]. In fact, this group obtained a CPD of 10.98 ± 0.86 and 16.73 ± 1.22 for human ADSCs expanded in 20% O_2 and 3% O_2, respectively[332]. In the present work and for the same passage, smaller values were obtained (4.5 ± 0.5 and 5.9 ± 4.6, respectively), even though the same tendency prevailed. The differences in the values might be due to the fact that herein, the

proliferation study started at P3 with cells previously thawed, while in the referred study it began at P0, therefore allowing the accumulation of PD from P1 and P2, with cell lines from a commercial source[332]. The hypoxic conditions were also slightly different, characterized by 2% O_2 in this work and 3% O_2 in the study by Estrada and colleagues (2012)[332]. This research group attributed the enhanced performance of ADSCs expanded in hypoxia to a smaller accumulation of ROS and a minor decrease in the telomere length throughout passages[332]. Authors found that an oxygen tension of 20% increased the rates of aneuploidy and double strand breaks per cell, as well as the number of cells affected, inducing an excessive DNA damage and unstable chromosomal aberrations[332]. A recent study by Oliveira and colleagues (2012), however, pointed out to the downregulation of the tumor supressor *p53*, the proto-oncogene *c-MYC*, and genes important for the DNA repair, namely *BRCA1*, by healthy BM-MSCs and ADSCs cultured under hypoxic conditions (2% O_2)[333]. Despite of maintaining the telomere length, hypoxic conditions induced microsatellite instability[333], which shows that it might be desirable to test other values of oxygen tension that protect the cells from excessive ROS, while affecting minimally the regulation of the referred genes.

A comparative study from Peng and co-workers in 2008 showed that ADSCs not only had a greater proliferation capacity but also grew faster than BM-MSCs[193]. The time ADSCs took to expand from P3 to P9 was approximately equal to healthy BM-MSCs, which is not concordant to what Peng and co-workers (2008) observed. As displayed in Figure IV-1 (graphics d and e), ADSCs expanded in normoxia were not superior to healthy BM-MSCs. Contrarily, when expanded in hypoxia conditions, ADSCs showed a behaviour concordant with that observed by the group of Peng (2008)[193], presenting higher CFI and CPD at any passage comparatively to healthy BM-MSCs, despite the absence of statistical significance. The group of Peng (2008) could also verify that ADSCs had significantly higher proportions of cells at the S phase, when compared to BM-MSCs, which could account for the higher proliferative potential of ADSCs in that study[193] in the case of hypoxia, herein. Although proliferating at a higher degree than healthy BM-MSCs, ADSCs were clearly overcame by AMI BM-MSCs in the case of the CFI (Figure IV-1, graphic d), even without a statistically significant difference. In the case of the CPD, the difference is slight, although showing the same tendency.

In the case of ignoring the donor M72A07, which was the youngest (35 years) and best donor (in the proliferative study) from the group of healthy BM-MSCs, than the results for the CFI and CPD from any oxygen tension are inferior to those of ADSCs. This fact points out that the number of samples used in each group of cells should be higher or involve donors with closer ages, in order to reach more consistent results.

Kern and colleagues (2006) also found supportive observations for a greater proliferative expansion potential for ADSCs, comparatively to healthy BM-MSCs, but only after P4[200]. This group noticed an inversion of the growing trend of the CPD at P5 and P6 for BM-MSCs and ADSCs, respectively[200]. In the present work, although this turning point did not have an impact in the CPD, it was also present, as previously mentioned, even though at different passages, namely at P4, P6, and P5 for healthy BM-MSCs, ADSCs, and AMI BM-MSCs expanded in normoxia conditions, respectively. The lower number of samples used in the present work might account for the low or even absent effect of the trend reversal (observed in the non-cumulative parameters) in the CPD. The authors

attributed the loss of proliferation of these cells to their senescence and verified that BM-MSCs had a higher senescence ratio at P2 than ADSCs[200]. The work of Dmitrieva and colleagues (2012) also supported the higher senescence effect for BM-MSCs, especially at P4[195]. This fact may be the reason why BM MSCs inverted their proliferative trend sooner than ADSCs, as equally observed herein for normoxia conditions. The senescence process could be the result of the oxidative stress referred by the study of Estrada and colleagues (2012), despite their observation of major impacts at higher cell passages (p15)[332]. Since hypoxia conditions enhance the resistance of ADSCs to the oxidative stress[332], it would be expectable a later turning point in the evolution of the PD for cells expanded in hypoxia conditions. Contrarily to the expectations, both types of healthy cells peaked the population doubling at P4 for expansion in hypoxic conditions (AMI cells reached the peak at P5), which also cancelled the difference between MSCs from healthy BM and AT observed in normoxia, herein and in the literature[200]. However, the absence of statistical significance for the PD and the low number of samples may have contributed to this unforeseen result.

Senescence was yet found, by the group of Tsai (2011), to be accelerated by normoxia conditions when compared to oxygen tensions of 1% in the expansion of BM-MSCs[334]. They observed a cessation of the proliferation at P6/P7 in normoxia conditions[334], which was similarly seen in the present work at the same passages, with more emphasis precisely for the BM cells. This higher sensibility of BM-MSCs to the oxygen tension may be due to the fact that their niche is hypoxic[216], whereby an oxygen tension superior to 1-6% might be enough to deregulate their performance which is adapted to a low oxygen tension. The authors referred yet that hypoxia conditions allowed the further expansion of the cells without a significant loss of the proliferation capacity[334]. Herein, a decrease in the PD could be observed also in the hypoxic condition, although at an apparent smaller rate. The absence of statistical significance could not, nonetheless, confer a more robust sustentation of these results. Following this decrease in proliferation, Tsai and co-workers (2011) also noted a significant increase of the expression of β-galactosidase and P21, which are associated to senescence, in the normoxia-expanded cells, and an increase of the proportion of hypoxia-expanded cells in the cell cycle[334].

The higher proliferative capacity achieved by AMI BM-MSCs in hypoxia when compared to ADSCs or healthy BM-MSCs might be derived from the two following factors:

- AMI BM-MSCs used in this work were extracted from younger donors. Ignoring the donor M55A11 AMI, with 56 years and the worst performance from this group of cells, the average age of the samples would be 45 ± 4 years, which would place these cells into the adult group (25-50 years), according to Zaim and colleagues (2012)[324]. Comparing to the healthy BM-MSCs, AMI cells could, therefore, suffer less consequences of age, thus justifying a resultant improvement in the proliferation rate when compared to healthy cells.
- After an AMI, a whole inflammation response is activated, as described in section I.2.2.1, and some important cytokines released in this context, namely TNF-α and IFN-γ[220; 226-230], might have an impact in the proliferation of MSCs. In a study by Prasanna and colleagues (2010), they concluded that the stimulation of BM-MSCs with TNF-α led to higher PD from P3 to P6, while an opposite trend was seen for the stimulation with only IFN-γ[335]. The combination of

both cytokines in the induction of MSCs for the evaluation of the secretory profile conducted to a decrease in the cell number, compared to non-inducted cells. Nevertheless, and given the results from Prasanna and colleagues (2010)[335], the situation that occurs *in vivo* is much more complex, therefore, the interaction of these and other inflammatory cytokines may translate into a higher proliferative potential for BM-MSCs of AMI patients, which would, then, explain the higher CFI and CPD obtained at the end of the present proliferative study, when comparing AMI BM-MSCs, expanded in a hypoxic condition, to the remaining cells.

In the normoxia conditions, the performance of AMI BM-MSCs is no longer better than the other cells, regarding the CPD. This observation is reverted if the donor M55A11 AMI is excluded from the analysis, which shows that more samples from different donors should be analysed in order to reach more robust values, not only in the group of AMI BM-MSCs but also in the healthy BM-MSCs and ADSCs groups.

As it was previously mentioned, some of the differences between the values obtained and the expected tendency might be explained by the set-up of the study. Since the moment at which the cell passages are performed is decided by the operator, who evaluates the confluence level and decides if the cells are ready for the passage, there is some variability at the cell passage point between the several cell lines studied in this work, especially at the beginning of the project, during which the operator was still consolidating skills and methods and was more prone to mistakes. Besides this, the type of Neubauer chamber used during this work was not always the same. For the majority of the healthy donor cells, a glass Neubauer chamber was used for the cell counts and this type of cytometer appeared to give an underestimation of the number of cells in the sample. For all the AMI BM-MSCs, disposable Neubauer chambers were available and their use was, therefore, preferred to accelerate the cell passage process. Nevertheless, Neubauer chambers appeared to give an overestimation of the number of cells and the counts were more variable between wells of the same Neubauer chamber. To avoid or minimize the impact of these factors into the results obtained, the proliferative study should be performed at fixed time points instead of being based on the operator's judgement, and an automatic cell counter should be used to obtain more reliable and consistent cell counts.

IV.2.2 Secretory Profile

The conjugation of TNF-α and IFN-γ has been used by several research groups to simulate an inflammatory environment *in vitro*[229; 230; 335]. These cytokines, which expression is induced by injury situations like AMI[317], are capable of regulating the expression of other cytokines, as well as changing the adhesion, migratory, and immunomodulatory properties shown by MSCs[230]. These facts motivated their use in the simulation of an inflammatory environment in the present work, in order to observe the changes of the secretory profile of MSCs for key components, such as IL-6, SDF-1, TGF-β, and VEGF. Therefore, and as described in section III.5, cells from P3 or P4 were exposed to IFN-γ and TNF-α for 4 days, under hypoxic or normoxic conditions, and the resultant concentration levels of the 4 aforementioned cytokines, secreted to the culture medium in response to the conditions tested, were

estimated using the respective ELISA kits and calibration curves obtained. The results for the SDF-1 and TGF-β ELISA kits were discarded since the kits could not detect any concentration level for those cytokines. The others are displayed in Figure IV-2 and Table IV-2.

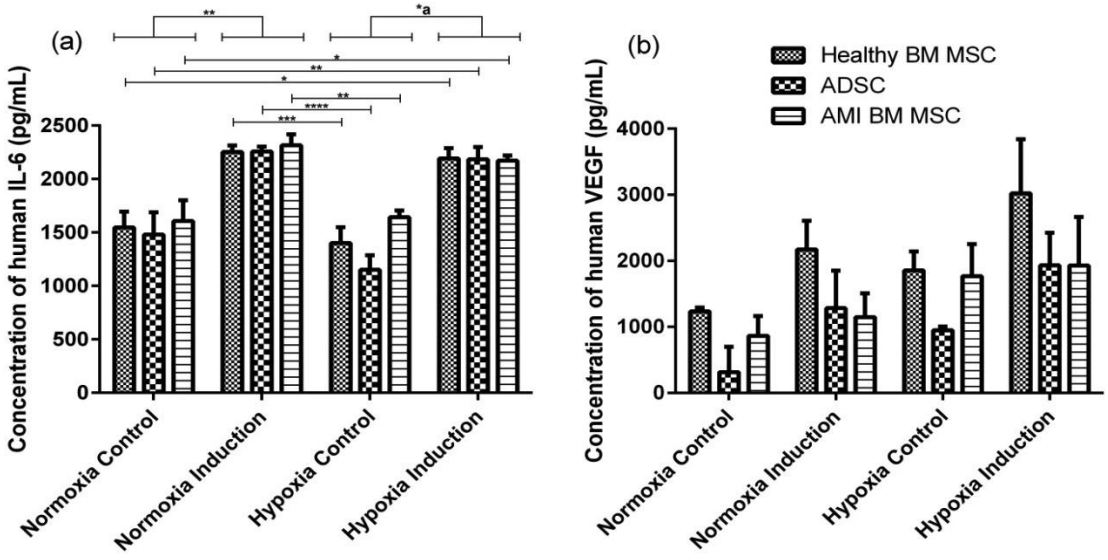


Figure IV-2. Secretory profile of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions, and stimulated or not with TNF-α and IFN-γ. (a) Concentration of human IL-6 (pg/mL); (b) Concentration of human VEGF (pg/mL). In both graphics, n = 3 for every type of cell and all the conditions studied with the exception of: healthy BM-MSCs in (a), where n = 2 for the normoxia control and hypoxia induction conditions, and in (b) but only for the condition of normoxia control; ADSCs in (b) for all the conditions. Results are presented as mean ± SEM. * p < 0.05; ** p < 0.01; *** p< 0.001; **** p < 0.0001; *a: p < 0.05 between the groups of AMI BM-MSCs, p < 0.01 between the groups of healthy BM-MSCs, and p < 0.0001 between the groups of ADSCs.

Table IV-2. Summary of the concentrations of human IL-6 and VEGF, in pg/mL, for healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions, and stimulated or not with TNF-α and IFN-γ. The ratios between normoxic and hypoxic (Norm/Hyp) values and between inducted and control (i/c) cells were also calculated. Results are presented as mean ± SEM. The statistical significance is indicated in Figure IV-2 and was omitted in this table to simplify its analysis.

		Concentration of the cytokine (pg/mL) (mean ± SEM)					
		IL-6			VEGF		
		Inducted	Control	Ratio (i/c)	Inducted	Control	Ratio (i/c)
Healthy BM-MSCs	Normoxia	2300 ± 62	1500 ± 148	1.46	2200 ± 436	1200 ± 58	1.76
	Hypoxia	2200 ± 97	1400 ± 148	1.57	3000 ± 821	1900 ± 287	1.63
	Ratio (Norm/Hyp)	1.03	1.10		0.72	0.66	
ADSCs	Normoxia	2300 ± 46	1500 ± 208	1.52	1300 ± 566	300 ± 382	4.06
	Hypoxia	2200 ± 114	1200 ± 136	1.90	2000 ± 494	900 ± 56	2.04
	Ratio (Norm/Hyp)	1.03	1.29		0.67	0.33	
AMI BM-MSCs	Normoxia	2300 ± 103	1600 ± 195	1.44	1100 ± 360	900 ± 300	1.33
	Hypoxia	2200 ± 48	1600 ± 61	1.32	2000 ± 735	1800 ± 484	1.09
	Ratio (Norm/Hyp)	1.07	0.98		0.60	0.49	

The secretory profile study made in this work was not well-succeeded for the analysis of SDF-1 and TGF-β. In fact, upon the treatment of the absorbance values obtained at the end of the experiment, the concentration levels estimated were null for every condition and type of cell analysed. This did not occur for the calibration curve and, at the same day these experiments were performed, IL-6 kit was also used, which displayed positive results for every condition analysed. Given these facts

and since the procedure was the same for all the kits, the probability of the failed results being a consequence of malfunctioning kits or operator's mistake is reduced. Instead, the concentration levels of these cytokines were possibly very reduced, thus an incubation period over-night should have been performed to enhance the signal.

In section I.4.4.6, some of the properties of IL-6 were mentioned, namely the role in the homing of MSCs into BM[236] and in the angiogenic effect[244]. The analysis of IL-6 (Figure IV-2 a) produced statistically significant results of varied levels when comparing the inducted values with the respective controls for both hypoxia and normoxia conditions and for the three types of cells. Indeed, higher concentrations of IL-6 were observed in the group subjected to induction with TNF-α and IFN-γ, when compared with the respective controls. Nonetheless, the hypoxia and normoxia control groups showed similar results, and so did also the inducted groups from both oxygen tensions, with no statistical differences in the referred pairs. The type of cells did not seem to produce substantial differences in the concentration of IL-6, which was perfectly manifested by the high inducted/control ratios and the almost unitary normoxia/hypoxia ratios, similar amongst the type of cells (Table IV-2).

In 2010, the group of Hemeda showed that non-stimulated BM-MSCs had a basal expression of IL-6 of approximately 3 ng/mL, value that would increase to almost 20 ng/mL with stimulation by TNF-α alone[230]. IFN-γ produced a negligible increase of IL-6, also when used alone[230]. A similar basal value for IL-6 was reported in the present work for both BM-MSCs and ADSCs, although the induction with both TNF-α and IFN-γ could only reach an increase to a maximum of 2.3 ± 0.103 ng/mL, observed for AMI BM-MSCs in normoxia conditions. This might be due to the fact that the impact of the stimulatory cytokines was not evaluated in an isolated way, like Hemeda and colleagues (2010) did[230], but together, and using lower concentrations (10 ng/mL of TNF-α and 500 U/mL of IFN-γ against 1 000 U/mL of both cytokines used by the referred research group[230]). Beyond this, the authors used cells seeded into 12-well plates and stimulated after reaching confluence[230], while in the present work, the cells are stimulated with the inflammatory cytokines 4 days after seeding into 2 pairs of wells of a 24-well plate and, by this time, they were not confluent yet. All these dissimilarities between studies may, therefore, affect the achieved concentrations of IL-6, albeit maintaining the general tendency of higher concentrations upon stimulation.

TNF-α was also capable of stimulating the production of IL-6 by ADSCs, as demonstrated by Lee and co-workers (2010), in a dose-dependent manner[317]. In fact, an addition of 10 ng/mL of TNF-α to the medium culture of confluent ADSCs, followed by an incubation of 48 hours, resulted in a secretion of almost 15 ng/mL of IL-6, compared to a value close to 0 ng/mL in non-stimulated cells[317]. These observations are, once again, supportive of the ones obtained in this work, since the induction with TNF-α and IFN-γ also led to a higher concentration of IL-6, from 1.5 ± 0.208 ng/mL in control cells to 2.3 ± 0.046 ng/mL in induced cells, for normoxia conditions. The lower inducted value achieved herein might, again, be a consequence of slight differences in the protocols, such as the cell density upon seeding (3 000 cells/cm^2 against 10 000 cells/cm^2 in Lee's work), moment of induction (4 days after seeding against confluence day), medium composition (10% FBS against low serum content), and time of stimulation (4 days against 48 hours)[317].

Kinnaird and colleagues (2004) observed the impact of hypoxic conditions in BM-MSCs and concluded that a low oxygen tension would augment the secretion of IL-6, from 3 885 pg/mg in normoxia to almost twice that value in hypoxia, approximately 7 665 pg/mg[268]. In the present study, the difference of concentrations between hypoxia and normoxia conditions was not statistically significant for any type of cell, which disagrees with the findings of Kinnaird and colleagues (2004). However, they used an oxygen tension inferior to the one applied in the present work (only 1% O_2[268]), a dissimilarity that might have impact in the expression of this particular cytokine. The details about the assay settings were not revealed, which hampers further comparisons with the assay performed herein.

VEGF has been shown to be angiogenic[243; 244] and to promote survival of MSCs in an ischemic and inflamed environment[261]. For the concentration levels of VEGF (Figure IV-2 b), no statistically significant differences were obtained. Nevertheless, inducted groups displayed increased concentrations than the respective controls, and the levels of VEGF appeared to be higher for hypoxia conditions, compared to normoxia conditions, both for the inducted and control groups. This is supported by normoxia/hypoxia ratios smaller than the unity (Table IV-2). The higher inducted/control ratios found for normoxia conditions fed the idea that cells expanded in normoxia conditions were more responsive to the treatment with stimulatory cytokines. In this case, ADSCs seemed to be the most responsive type of cells, even in hypoxia conditions. It is also noteworthy that for ADSCs the control levels of VEGF were the smallest amongst the three types of cells, which might explain the higher inducted/control ratios. Though ADSCs had the greater response to the induction with IFN-γ and TNF-α, healthy BM-MSCs were the cells reaching the highest concentration values of VEGF.

In 2008, the group of Peng performed a cytokine expression comparison by real-time RT-PCR between MSCs from different sources and discovered that basal levels of VEGF were higher for BM-MSCs than for ADSCs[193], which could also be observed herein for healthy BM-MSCs, although through a different analysis (ELISA) and without statistical significance. The study by Li and co-workers (2012) confirmed the increased expression of VEGF in BM-MSCs compared to ADSCs, 3 ng/mL against approximately 2.5 ng/mL, respectively, numbers much superior to those found in this work, possibly due to a larger number of cells seeded (100 000 cells/mL)[336]. The already mentioned study by Kinnaird and colleagues (2004) could also provide evidences of the stimulation of hypoxic conditions towards an increased expression of VEGF in BM MSCs, with the normoxia/hypoxia ratio achieving a value of approximately 0.54[268], similar to the values obtained herein, 0.66 for healthy BM-MSCs and 0.49 for AMI BM-MSCs. For ADSCs, a similar trend was observed by Rehman and colleagues in 2004[243]. Authors reported an increase of approximately 5-fold for the secreted amounts of VEGF, from normoxia to hypoxia conditions[243], while herein an almost 3-fold increase was observed (the inverse of the 0.33 normoxia/hypoxia ratio for these cells in non-stimulated conditions).

In 2013, Melief and colleagues reported the effect of the induction of ADSCs and BM-MSCs with 500 U/mL of IFN-γ: slightly increased concentrations of VEGF in BM-MSCs and higher concentrations of IL-6 for the ADSCs[337]. ADSCs presented, in fact, a greater secretion of other cytokines, which was hypothesized by the authors to be due to a higher metabolic activity displayed by

these cells[337]. While the VEGF tendency supports the findings of the present work, the same cannot be stated for the IL-6 difference between cells. The observations of that study are also discordant to the lack of effect in the concentration of this cytokine induced by the use of IFN-γ, stated by Hemeda[230], although it could be related with differences in the protocol, namely at the concentrations of IFN-γ used[230; 337]. It is also noteworthy that study by Melief and co-workers (2013) focus only in the effect of IFN-γ[337], while herein, the conjunct action of that factor and TNF-α was evaluated and could lead to a lack of differences between the performance of the three type of cells studied.

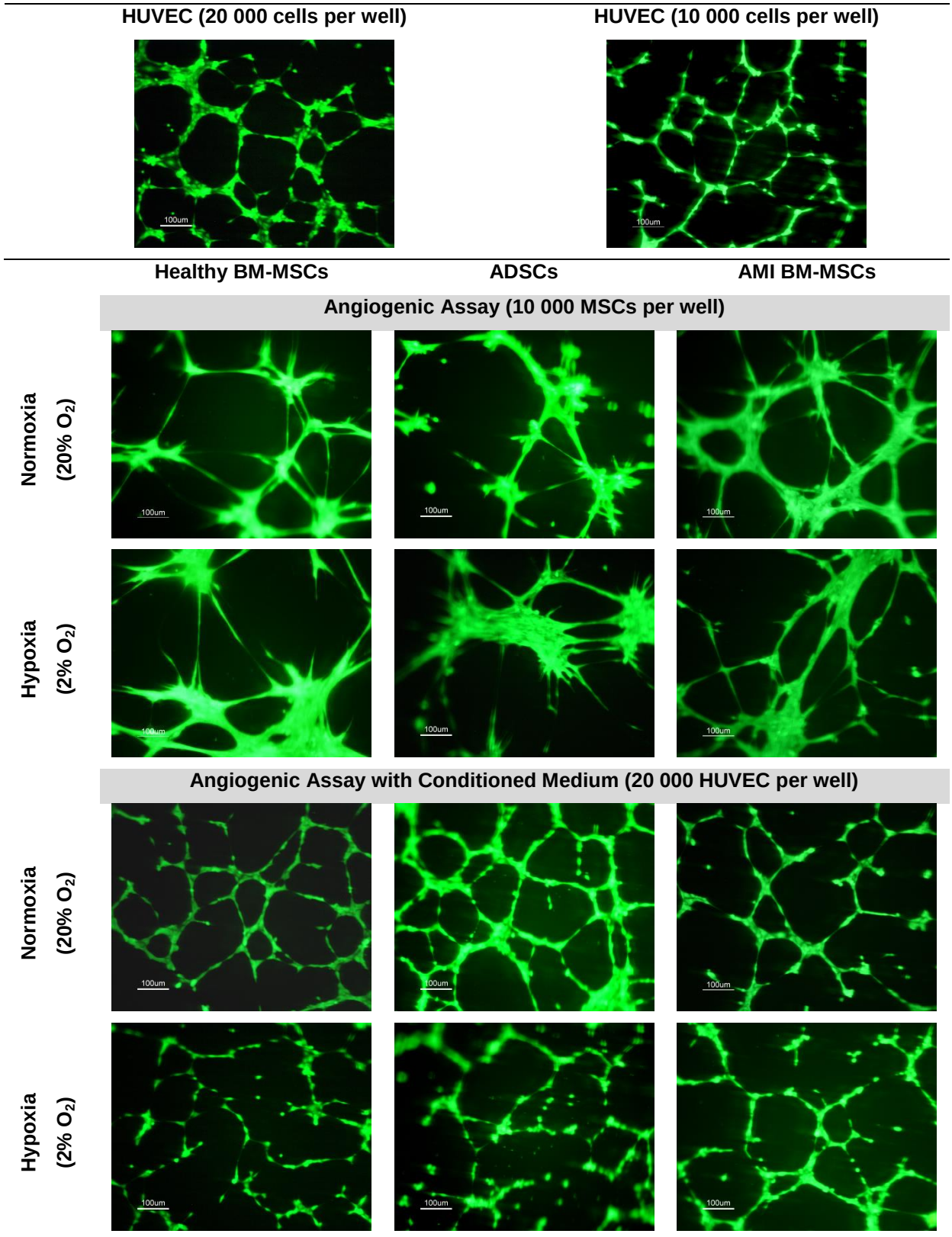
Analysing the results for the group of BM-MSCs, both healthy and AMI cells presented a similar performance in normoxia and hypoxia conditions, in the absence of induction. When subjected to the action of TNF-α and IFN-γ, the healthy cells could secrete higher levels of VEGF, even without a statistically significant difference from AMI cells, while the AMI BM-MSCs presented a similar profile to the one of ADSCs. This points to the possibility that previous exposure of BM-MSCs to these inflammatory cytokines in the sequence of an AMI episode saturates their answer in a posterior *in vitro* situation. However, that would also be supported by a higher VEGF basal expression in these cells, which was not observed. A higher number of samples should be used in the attempt to reach statistically significant differences in the performance of the three types of cells, thus allowing the inference of more robust conclusions.

IV.2.3 *In vitro* Angiogenic Potential and Tube Incorporation Potential

Several studies have highlighted the impact of MSCs in angiogenesis and the importance of this mechanism in the improvement of the heart condition after the AMI[258; 267-270]. To exert such factors, MSCs might secrete angiogenic factors like HGF[243], VEGF[243; 244], and IL-6[244], integrate the new tubes that are being created by ECs[338], or differentiate and give rise to capillary-like networks[336; 339; 340]. To test these different mechanisms and the influence of cell source and oxygen tension, three different settings were performed, as previously described in section III.6: an angiogenic assay, where 10 000 MSCs were seeded per well, an angiogenic assay with conditioned medium, where 20 000 HUVEC were seeded per well in conditioned medium from MSCs culture, and a tube incorporation assay where the co-culture of HUVEC and MSCs, in a proportion of 20 000 : 5 000 was tested. In all the settings, HUVEC cultured alone were used as a positive control. The seeding was done into Matrigel, a mixture of ECM and basement membrane proteins derived from mouse, which stimulates the *in vivo* process of attachment, migration and differentiation of ECs into tubular structures[341]. The culture medium used was EGM-2, which is optimized for the rapid proliferation of HUVEC and contains VEGF and FBS. For the co-culture experiment, MSCs were previously incubated with Dil-Ac-LDL to allow their identification in the tube-like structure formed by HUVEC. Acetylated LDL is a specific marker of differentiation of cells forming capillary-like structures and, if positive, shows that MSCs suffered that differentiation and were integrated into the structure[342].

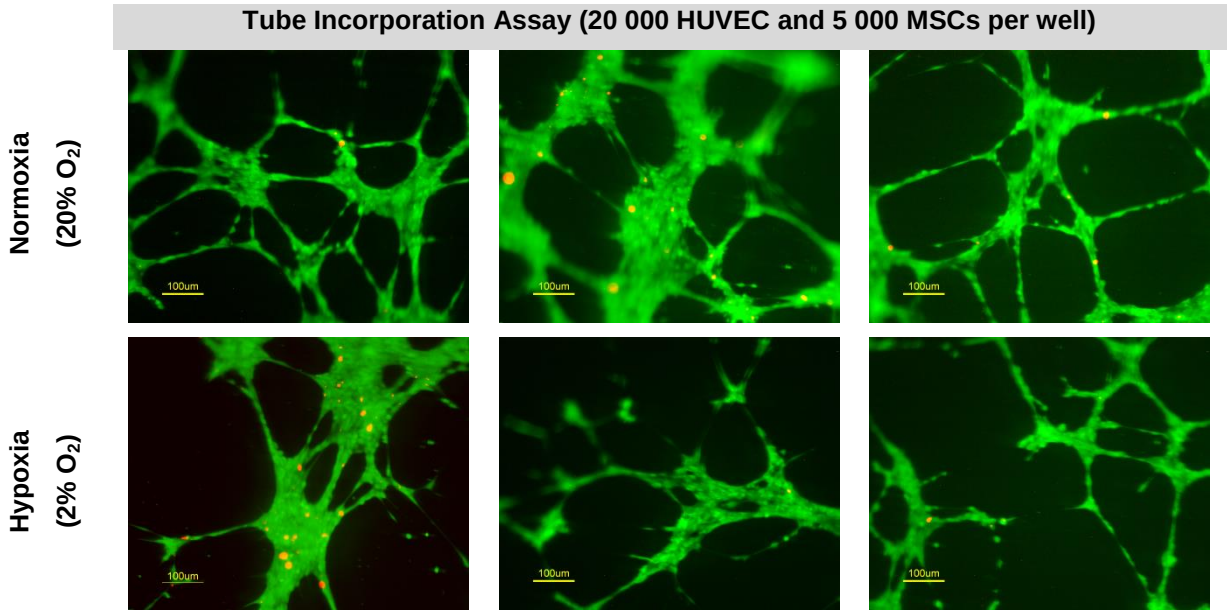
The numbers used in this work for the MSCs seeded alone or in co-culture with HUVEC, as well as the incubation time, were previously optimized. To compare the results obtained for each condition, the number of tubes, length of tubes, and number of connections was evaluated. The results of this assay are displayed at Figure IV-3 and Figure IV-4.

Figure IV-3. Fluorescence images from the angiogenic assay, angiogenic assay with conditioned medium, and tube incorporation assay, of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions. For all the conditions studied, the number of samples analysed was 3, except for angiogenic assay with condition medium, where n = 1 for healthy BM-MSCs, and n = 2 for ADSCs. The images were chosen from the several donors in order to be representative of the average values obtained for each condition and presented in Figure IV-4.



(To be continued)

(continuation of Figure IV-3)



Analysing the results presented in Figure IV-3, clear differences are observed, especially between the positive controls and the results from angiogenic assay and tube incorporation assay. Regardless of the oxygen tension and the type of cells, and comparing to the respective positive controls, MSCs seeded alone were more prone to form larger junctions from which there was a divergence of many tubes. Some tubes were also thicker than the ones present at HUVEC cultures. These differences might be a consequence of the larger size of MSCs when compared to HUVEC, which was observed at the microscope. During the process of optimization of this setting, a higher number of MSCs almost exclusively resulted in large cellular agglomerations. 10 000 cells per well originated best results after a careful homogenization of the cells to avoid their concentration at the centre of the wells.

Comparing the angiogenic assay with conditioned medium to the positive control, the tubular network formed was much more similar than in the case of angiogenic assay with MSCs, albeit the presence of many incomplete tubes in this setting, more evident in BM-MSCs from both healthy and AMI donors, and hypoxia conditions. The tubular network had also a more regular pattern, similarly to that observed for the control.

The tube incorporation assay was optimized relatively to the ratio of HUVEC and MSCs. A ratio of 20 000 : 10 000 induced the formation of agglomerations, while ratios of 15 000 : 5 000 and 15 000 : 1 000 seemed to be inefficient for the formation of tubes. The ratio that was found to be the most adequate, 20 000 : 5 000, gave rise to structures similar to the ones observed in the angiogenic assay of MSCs, with thick branch points and larger tubes. The orange spots in the green tubular network correspond to MSCs that successfully integrated into the tubular structure of the HUVEC.

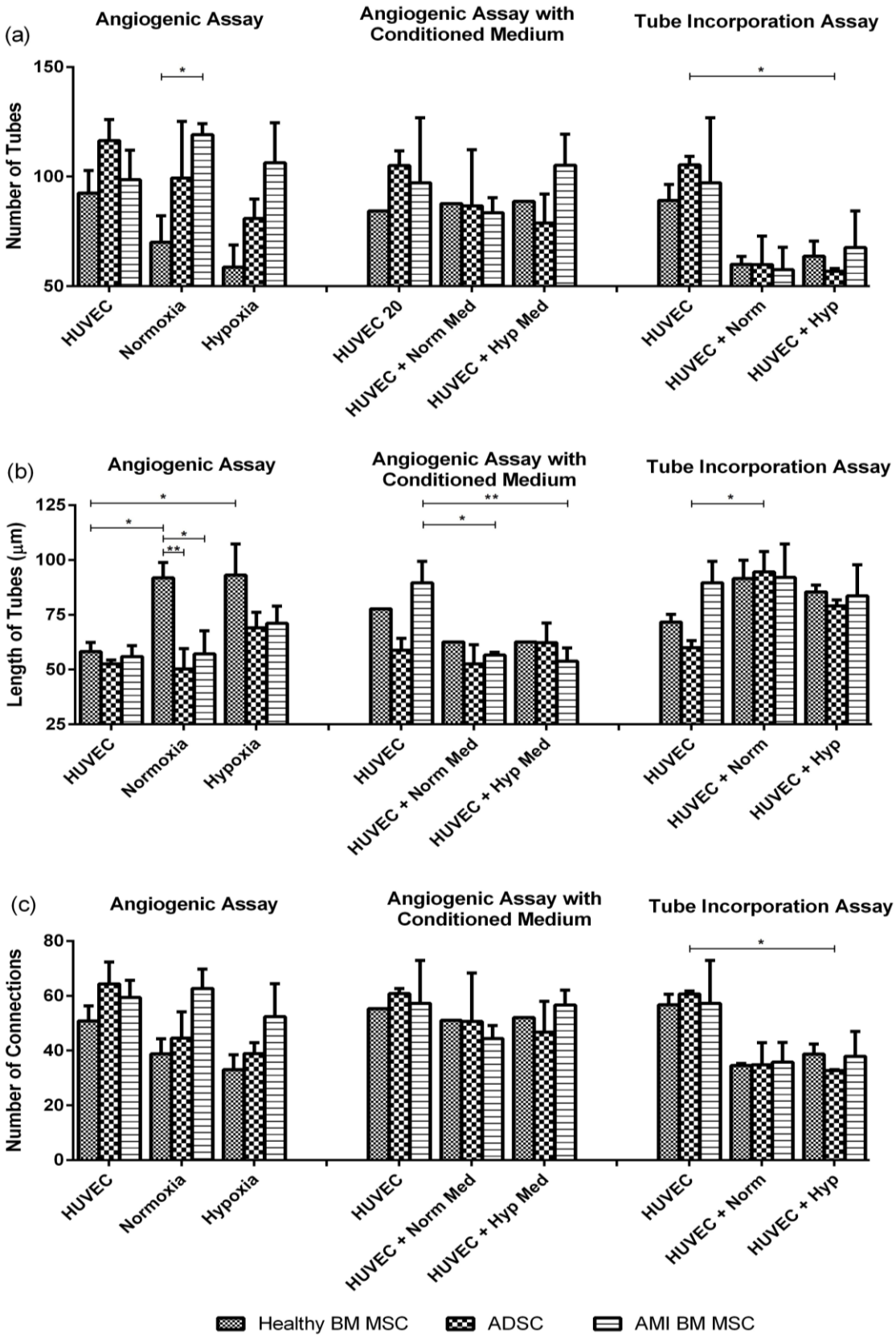


Figure IV-4. Results of the angiogenic assay, angiogenic assay with conditioned medium, and tube incorporation assay, of healthy BM-MSCs, ADSCs, and AMI BM-MSCs under normoxic (20% O_2) or hypoxic (2% O_2) conditions. (a) Number of tubes; (b) Length of tubes (µm); (c) Number of connections. In all the graphics and for all the conditions studied, the number of samples analysed was 3, except for angiogenic assay with condition medium, where n = 1 for healthy BM-MSCs, and n = 2 for ADSCs. Results are presented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$.

IV.2.3.1 Angiogenic Assay

In the angiogenic assay (Figure IV-4, first group of results in a, b, and c), the positive control used (10 000 HUVEC per well) was consistent amongst experiments for the different parameters estimated. It is noteworthy to refer that each sample analysed in this work was subjected to this group of assays in different days and using HUVEC from different passages. The fact that the number of tubes, length of tubes and number of connections was similar for all the experiments highlights the reproducibility of the assays for the HUVEC.

Relatively to the MSCs used herein, different results were observed, particularly at the number of tubes. In fact, healthy BM-MSCs and ADSCs presented lower number of tubes when compared to the respective HUVEC control groups, both in normoxia and hypoxia conditions. The number of tubes for ADSCs was, however, higher than for healthy BM-MSCs. In the case of AMI BM-MSCs, these cells had an increase number of tubes than HUVEC, for both oxygen tensions. The difference in the number of tubes was statistically significant ($p = 0.0445$) between AMI BM-MSCs and healthy BM-MSCs, respectively 120 ± 5.1 versus 70 ± 12.2. The general trend for this parameter was a crescent number of tubes from healthy BM-MSCs, to ADSCs, and finally to AMI-MSCs, and decreased values in hypoxia, compared to normoxia.

Regarding the length of the tubes, HUVEC, ADSCs and AMI BM-MSCs cultured in normoxia presented similar values, variable between 50 ± 9.2 μm and 60 ± 4.2 μm. A non-statistically significant difference was observed between HUVEC and that same group of cells, but expanded in hypoxia, which had tubes with a length of approximately 70 ± 7.1 μm to 70 ± 7.8. Statistical differences were obtained, nonetheless, when comparing healthy BM-MSCs to the respective HUVEC group ($p = 0.0243$), to ADSCs ($p = 0.0042$), and to AMI BM-MSCs ($p = 0.0155$), all expanded in normoxia conditions, and when comparing healthy BM-MSCs from hypoxia conditions to HUVEC ($p = 0.0194$). However, healthy BM-MSCs presented very similar values in both oxygen tensions, between 90 ± 7.0 μm and 90 ± 14.2 μm. It seems that a lower number of tubes is, therefore, associated to longer tubes.

For the number of connections, there was a slight difference between the cells, following the same tendency observed for the number of tubes (Figure IV-4 c). AMI BM-MSCs were the only cells presenting a similar number of connections as their control group, varying between 50 ± 12.1 and 60 ± 7.1, with a minor decrease in the hypoxia cultured cells. For the remaining types of cells, the number of connections was inferior to the respective control groups, with ADSCs presenting more connections than healthy BM-MSCs. Also for these types of cells, hypoxia conditions led to a slight reduction in the number of connections.

Comparatively to the other assays, the angiogenic assay was more efficient in highlighting differences between cell types and gave rise to more statistically significant dissimilarities, not only between cell types but also comparing to the control groups.

In 2011, the group of Eto used a cocktail with basic bFGF, Epidermal Growth Factor (EGF), PDGF and TGF-β to induce ADSCs and observe the effect on the network formation[340]. Comparing to HUVEC (in which the induction did not seem to cause any impact), treated ADSCs presented a higher total network length, a more complex capillary-like network structure, and amongst the cocktail of factors, bFGF was the most relevant for the assay[340]. Although such induction was not performed in

the present work, it was already mentioned in IV.2.2 that a hypoxic condition was capable of increasing the secretion of VEGF and, as shown by the group of Kinnaird (2004), the low oxygen tension also stimulated the higher production of bFGF[268]. Making a parallelism between the induction cocktail with synergistic effects of the group of Eto (2011)[340] and the hypoxic conditions used herein, both strategies contributed to the great increase of the tube length when compared to HUVEC controls, although herein the difference between hypoxia and normoxia for the healthy BM-MSCs was minimal, since the tubes were already quite long for the normoxic cells.

In 2012, Janeczek Portalska and colleagues compared the angiogenic capability of BM-MSCs with HUVEC and concluded than HUVEC presented a higher total tube length (close to 50 cm for HUVEC and approximately 40 cm for MSCs) and more branching points than MSCs (100 against 80 branching points, respectively)[339]. Herein, a smaller number of connections was obtained for the same cells (50 ± 5.4 for HUVEC and 40 ± 5.5 for healthy BM-MSCs), although the tendency of having more connection points in HUVEC matched the findings of Janeczek Portalska and co-workers (2012)[339]. Nevertheless, the total length of the tubes of MSCs was estimated to be higher than HUVEC (6.4 mm against 5.3 mm), contrarily to the observations of the group of Janeczek Portalska (2012)[339]. The differences in the order of magnitude of the values and even the contrary trend of the total tube length between the present study and the one by Janeczek Portalska and co-workers (2012) might be due to the differences in the setting of the angiogenic assay, since the group of Janeczek Portalska not only seeded larger wells (6 well-plate wells against 96-well plate wells herein) but also allowed an incubation for a longer period (24 hours against 8 hours)[339], which might have benefited the tubular network of HUVEC.

In their study from 2012, Li and colleagues compared several types of cells in the context of myocardial repair and concluded that ADSCs gave rise to longer tubes than BM-MSCs, albeit without a statistically significant difference between them[336]. In this study, authors could also verify that ADSCs had an increased production of important growth factors, namely angiopoietin-2, bFGF, and IGF-1, albeit other factors, such as HGF, SDF-1, and VEGF were augmented in the case of BM-MSCs[336]. In the present work, an opposite trend was obtained concerning the length of the tubes formed in Matrigel, with BM-MSCs giving rise to less, but statistically significant longer tubes than ADSCs, accompanied by a higher production of VEGF in normoxia and hypoxia.

IV.2.3.2 Angiogenic Assay with Conditioned Medium

In the angiogenic assay with conditioned medium from the culture of MSCs (Figure IV-4, middle group of results in a, b, and c), the values of the studied parameters where slightly more variable for the HUVEC groups, albeit with no statistical significance.

The results for the number of tubes, length of tubes and number of connections were consistently similar amongst oxygen tensions and the three conditioned medium-exposed HUVEC groups, with no statistically significant differences. Relatively to the number of tubes, this parameter presented similar values when comparing HUVEC control groups to HUVEC cultured in the conditioned medium from healthy BM-MSCs, expanded in both normoxia and hypoxia conditions, with values of approximately 84, 88 and 89 tubes, respectively. In the case of HUVEC cultured in the

conditioned medium from ADSCs, HUVEC had slightly more tubes (110 ± 6.7) than conditioned cells in both oxygen conditions (from 90 ± 25.7 in normoxia to 80 ± 13.2 in hypoxia). HUVEC cultured in the conditioned medium from AMI BM-MSCs were the only group of cells displaying an increased number of tubes than the positive control, but only in hypoxia (110 ± 14.1 versus 100 ± 29.7, respectively).

As aforementioned, the average length of the tubes did not differ considerably between the conditioned HUVEC groups and the respective control, varying between approximately 50 ± 8.8 μm and 60 μm. The exceptions were the control groups for the healthy BM-MSCs conditioned medium and AMI BM-MSCs conditioned medium, presenting values of approximately 80 μm and 90 ± 9.7 μm, respectively. The fact that HUVEC cultured in either type of BM-MSCs conditioned medium had smaller tubes than the control is in agreement with the observations that several tubes from these two conditioned medium seemed incomplete, as it can be seen in Figure IV-3. A significant difference was also observable between the length of the HUVEC control group and the HUVEC cultured in the AMI BM-MSCs conditioned medium, in both normoxia ($p = 0.0153$) and hypoxia ($p = 0.0097$). The oxygen tension did not seem to produce differences in the length of the tubes.

The number of connections in the control groups is overall higher than in the HUVEC expanded in conditioned medium groups, regardless of the oxygen tension. This is supportive of the images presented in Figure IV-3, since both an interruption in the middle of the tubes and an incomplete connection between tubes could be seen. The differences were higher for ADSCs conditioned medium, with the control group presenting 60 ± 1.8 connections, against 50 ± 17.7 and 50 ± 11.3 for normoxia and hypoxia conditions, respectively. For the AMI BM-MSCs conditioned medium, both the control group and the HUVEC in the hypoxic medium presented a similar number of connections, 60 ± 15.7 and 60 ± 5.5, a number that fell to 40 ± 4.7 in the normoxic conditions.

This assay produced the most similar results to the controls, as supported by the images displayed at Figure IV-3, and had no capacity to highlight differences between the types of cells.

In the study by Zhang and colleagues (2013), the conditioned medium of BM-MSCs was capable of inducing an increase in the length of the tubes formed by HUVEC, when compared to HUVEC cultured in base culture medium (α-MEM)[343]. In the present work, conditioned medium from BM-MSCs from both healthy and AMI donors induced the formation of shorter tubes, in an opposite trend to the reported by the referred research group[343]. Nevertheless, the controls herein corresponded to HUVEC cultured in EGM-2, which is a more adequate medium for the expansion of HUVEC and might be, therefore, more efficient in inducing the formation of the tubular structure, even in the absence of the extra factors present in the conditioned medium of MSCs, than α-MEM. It is then hypothesised that in the study by Zhang and co-workers (2013)[343], the controls used were negative, while herein were positive, and this has an impact in the interpretation of the results.

Using MSCs from rats, the group of Rahbarghazi (2013) performed three experiments similar to the ones in this work, a single cell culture (correspondent to the angiogenic assay herein), a co-culture system (correspondent to the tube incorporation assay) and a conditioned medium-dependent culture (correspondent to the angiogenic assay with conditioned medium)[344]. Relatively to the single cell culture, BM-MSCs were not capable of forming tube-like structures[344]. Instead, large aggregates formed[344], and based on the results obtained in the work during the optimization process, this might be

explained by the excessive number of cells per well (20 000 instead of 10 000 cells) and the use of an inadequate medium to support the formation of tubular structures (DMEM-low glucose instead of EGM-2 or similar). For the conditioned medium assay, they observed a statistical significant increase in the tube length of ECs subjected to the conditioned medium from BM-MSCs compared to ECs in DMEM-low glucose[344]. Herein, the tube length of HUVEC tubes did not increase in the presence of conditioned medium from BM-MSCs, although there was not a statistical significant difference in the values obtained. Nevertheless, the type of control used for the comparison of the results might explain this divergence. In fact, the control used by the group of Rahbarghazi (2013)[344] is thought to be negative since they did not use a medium adequate to expand ECs, while herein, the control is positive. Relatively to the findings for the co-culture assay, the results will be discussed in IV.2.3.3.

Another study, from the group of Hung (2007), demonstrated that the length of human aortic ECs incubated in hypoxic conditions was slightly smaller in the control medium (fetal bovine serum-free endothelial basal medium) than in normoxic conditioned medium from BM-MSCs cultured for 2 days, and greatly increased in hypoxic conditioned medium[244]. In the present work this was not observed for neither the two types of BM-MSCs condition media nor oxygen tensions, with HUVEC controls presenting statistically significant longer tubes than HUVEC cultured with AMI BM-MSCs conditioned medium. Nonetheless, the type of ECs, and the time MSCs were cultured to produce the conditioned medium (1-4 days herein, against 2 in the work of Hung and co-workers, 2007[244]) might explain the absence of a positive effect upon the use of conditioned medium. Beyond this, only one sample could be used in the healthy BM-MSCs conditioned medium, and so the results obtained in this group might not be representative of the action of healthy BM cells in the formation of tube-like structures by HUVEC. Instead of observing an increase in the length of tubes, the conditioned medium from AMI BM-MSCs cultured in hypoxia induced a higher number of tubes when compared to the control or to the HUVEC subjected to the correspondent normoxia medium. Even without a statistical significance, this finding points to an effect, even if subtle, in the angiogenic property of HUVEC. As shown in IV.2.2, VEGF levels were increased in a hypoxic situation for both healthy and AMI BM-MSCs and, as seen by Hung and colleagues (2007), this and other cytokines are up-regulated by hypoxic conditions in MSCs[244]. The combined effect of those factors were crucial for the activation of the PI3K-Akt which, in turn, was responsible for the apoptosis inhibition, increased cell survival, and enhanced tube formation[244]. The blockage of VEGF or IL-6 through the use of antibodies did not hamper the effects of the hypoxic conditioned medium, which shows that the important aspect in this context is the combined actions of the secreted factors, instead of an isolated role of each one[244].

In 2013, the group of Liu compared the effect of oxygen tension in the formation of tubular structures by HUVEC[345]. Using the conditioned medium of ADSCs grown in hypoxia (1% O_2) or normoxia (21% O_2) for 48 hours, they observed that HUVEC presented a higher total network length when incubated with the hypoxic medium than with normoxic medium[345]. In the present work, the length of the tubes cultured with hypoxic ADSCs conditioned medium was only faintly higher than normoxia or control groups, with no statistical significance. Nevertheless, and as it was pointed out in IV.2.2, hypoxia conditions induced, in fact, higher levels of VEGF, although ADSCs were the type of cells with the lowest levels of this growth factor, comparatively to BM-MSCs. The authors also verified

that the use of neutralizing antibodies against VEGF and bFGF led to a reduction in the length of the tubes[345], contrarily to the findings of Hung and colleagues (2007)[244].

The research group of Zhang showed, in 2012, that the conditioned medium of BM-MSCs, cultured for 48 hours in hypoxia conditions, was significantly more efficient in inducting HUVEC to form tubes than the conditioned medium from normoxia conditions[346]. With the conditioned medium from low oxygen tensions (1% O_2), the number of tubes formed was almost 3-fold and 8-fold higher than with normoxia conditioned medium or conditioned medium from HUVEC expanded in normoxia[346]. Herein, the number of tubes formed by HUVEC in conditioned medium from healthy BM-MSCs was comparable to the control, regardless of the oxygen tension used to produce the conditioned medium. However, the conditioned medium from AMI BM-MSCs expanded in hypoxia conditions gave rise to a higher number of tubes when compared to the respective control, despite the lack of statistical significance. This absence of a significant improvement in the number of tubes upon culture with a hypoxic conditioned medium may be explained by a shorter time of incubation that HUVEC were subjected herein (8 hours against 48 hours in the work of Zhang and co-workers, 2012) or, in the case of healthy BM-MSCs, by an insufficient number of samples to represent the trend in this cell population. The authors also verified that the hypoxic conditioned medium was almost 2-fold richer in VEGF and IL-6 than the normoxic one, supporting the greater angiogenic effect[346]. Herein, although there were no differences between the expression of IL-6 in normoxia or hypoxia conditions, the concentration of VEGF was approximately 1.5- and 2.0-fold higher for healthy and AMI BM-MSCs, respectively, expanded in hypoxia, which is, therefore, consistent with the slight improve in the number of tubes observed for AMI conditioned medium.

IV.2.3.3 Tube Incorporation Assay

In this setting (Figure IV-4, later group of results in a, b, and c), and with the exception of the positive HUVEC control group for AMI BM-MSCs, which showed longer tubes than the rest of the controls, the controls displayed very similar results amongst them.

A markedly reduction in the number of tubes was observed from the control group to the co-culture groups in either normoxia or hypoxia conditions, from 90 ± 7.3 – 110 ± 3.9 tubes to 60 ± 1.2 – 70 ± 16.7 tubes, respectively. This decreasing was statistically significant in the comparison of the co-culture of HUVEC with ADSCs (expanded in hypoxia) and the respective control group ($p = 0.0451$).

Associated to this drop of the number of tubes was an increase in the length of the tubes in the co-cultures. In fact, both the co-cultures of healthy BM-MSCs and ADSCs had longer tubes than the respective control groups, with a small reduction in the case of hypoxia, comparing to normoxia conditions. For the co-culture of ADSCs, this relative increase was statistically significant ($p = 0.0355$), from approximately 60 ± 3.3 μm in the control group to 100 ± 9.4 μm in the normoxic co-culture. Nevertheless, for the co-culture with AMI BM-MSCs, the values between the control group and the co-cultures were very similar, varying from 80 ± 14.3 μm to 90 ± 15.2 μm.

The number of connections was quite homogeneous amongst co-culture groups (between 30 ± 0.4 and 40 ± 3.6), and quite lower than the respective controls (between 60 ± 15.7 and 60 ± 1.1).

There was a statistically significant difference between the control and the co-culture with ADSCs expanded in hypoxia.

This assay produced results quite different from the ones of the angiogenic assay with conditioned medium, namely by giving rise to inferior amount of tubes, but longer, with less connections, and higher complexity than the ones formed upon conditioned medium.

As previously referred in IV.2.3.2, the group of Rahbarghazi (2013) performed a co-culture system to evaluate the effect of MSCs in the formation of tubular structures, together with ECs, using a ratio of 1:4[344]. In this case the authors obtained a statistically significant increase in the length of the tubes formed by the co-culture when compared to ECs alone[344]. Unlike the results of the conditioned medium, the co-culture of HUVEC with healthy BM-MSCs in the same ratio also gave rise to longer tubes. Despite the already mentioned differences in the setting of the experiment between this work and the work of Rahbarghazi and colleagues (2013)[344], it should be highlighted that the positive results obtained herein were also extended to ADSCs (with a statistically significant increase of the length in the case of the co-culture with this cells), and the effect of a co-culture may be more robust than the simple use of the condition medium of the cells. In fact, according to the group of Duffy (2009), the co-culture of MSCs with ECs provided a stabilization of the tubular structure that was not similar to the effect induced solely by conditioned medium[338]. They also observed that the co-culture with BM-MSCs resulted in longer tubes than the ones seen in an endothelial cell culture alone[338], which supports the findings of this work. In spite of giving rise to a decreased number of tubes when compared to the respective controls or even the conditioned medium groups, the co-cultures, particularly the ones with healthy BM-MSCs and ADSCs, induced longer tubes in both normoxia and hypoxia, although the later were slightly smaller. The group of Duffy reported yet an increase in both the thickness of the vessel-structure and the size of the junctions, similarly to what was obtained herein (Figure IV-3), for the co-culture systems, hypothesizing the direct cellular contact as an important mechanism by which MSCs contributed to the formation and stabilization of the vessels[338].

IV.2.4 Oxidative Stress Resistance Potential

The capacity of the cells to resist stressing imposed by ROS is quite relevant in the context of an AMI. The inflammation response that is triggered after the ischemic event and the reperfusion techniques used to re-establish the blood flow increase dramatically the burden of ROS[17; 18], which are damaging to the CMCs[347] and to the cells that are transplanted into the injured site in a cellular therapy[261]. The higher survival of the implanted cells to this aggressive ischemic environment might improve the outcome of the cellular treatment for this disease[256]. Therefore, several strategies, from incubation of the cells in hypoxic conditions[242; 261] to the preconditioning with growth factors[262; 263] have been investigated. To test the effect of source and oxygen tension in the survival rate of MSCs, an oxidative stress resistance assay, based on the induction with H_2O_2, was performed according to the protocol described in section III.7.

This assay suffered a long process of optimization, in which all the parameters were tested, namely the medium culture and percentage of FBS, the initial cell density, the time cells were cultured before being inducted with H_2O_2, the concentration of H_2O_2 used in the induction, and the time of

incubation with H_2O_2. Even with all this variables tested, the majority of the results obtained, especially for the CytoTox 96® Non-Radioactive Cytotoxicity Assay, were unreasonable, thus, they will not be shown in this work. Instead, a discussion of the optimization process will be done, and some results from the literature will be presented as expected results for the samples herein studied.

In Table IV-3, a summary of the several stages of optimization of the assay is displayed, with a brief indication of the issues encountered.

Table IV-3. Process of optimization of the oxidative stress resistance quantification assay performed in the present work. FC – Flow Cytometry.

Optimization	Culture Medium	Initial Cell Density (cells/cm^2)	Time in Culture Before Induction	Concentration of H_2O_2 (µM)	Incubation Time with H_2O_2	Problems
1	DMEM + 10% FBS	3 000	4 days	10 000	24 h	Almost all cells died upon H_2O_2 induction (impossible to count viable cells; undefined population in FC results); high background in LDH assay (small differences between induction and control)
2	DMEM + 10% FBS	3 000	4 days	10 000	2 h	Almost all cells died upon H_2O_2 induction (impossible to count viable cells; undefined population in FC results); high background in LDH assay (negative values)
3	α MEM + 10% FBS	3 000	4 days	10 000	2 h	Almost all cells died upon H_2O_2 induction (impossible to count viable cells; undefined population in FC results); still high background in LDH assay (negative values)
4	α MEM + 10% FBS	3 000	4 days	100	24 h	Cells grew at different rates in inducted and control wells (negative values in LDH assay); Still high background in LDH assay (small differences between induction and control, unreasonable results)
5	α MEM + 10% FBS	6 000	1 day	100	24 h	Cells grew at different rates in inducted and control wells (negative values in LDH assay); Still high background in LDH assay (small differences between induction and control)
6	α MEM + 10% FBS	6 000	1 day	200	6 h	Still high background in LDH assay (small differences between induction and control)
7	α MEM + 5% FBS	6 000	1 day	200	6 h	Still high background in LDH assay (small differences between induction and control, unreasonable results)
8	α MEM + 5% FBS	6 000	1 day	100	24 h	Still high background in LDH assay (small differences between induction and control, unreasonable results)
9	α MEM + 10% FBS	6 000	1 day	100	48 h	Still high background in LDH assay (small differences between induction and control)

One of the problems faced during the optimization of this assay was the concentration of H_2O_2 used to induct an oxidative stress in the cells. The extremely high value used in stages 1-3, 10 mM instead of 100 µM as initially planned, was actually not wilful but a result of miscalculations concerning the preparation of the H_2O_2 solution. Nevertheless, it was possible to see a total response of the cells to this extremely high concentration of H_2O_2. When observed at the optical microscope, almost all the cells were in suspension, an indication of cell death, even when the incubation time was reduced from 24 hours to only 2 hours. In the flow cytometry results for the FITC Annexin V staining, the identification of a population of MSCs was not possible. Instead, there was a blur correspondent to particles of small dimensions, resultant from the death process of the cells during the incubation with

H_2O_2. Despite the massive cell death that was clear in the optical microscope and the flow cytometry analysis, the results for the LDH release were only concordant with these findings when the incubation time was 2 hours (stage 2 of the optimization process). In this case, the non-normalized cytotoxicity reached 90% and 40% for inducted cells, in normoxia and hypoxia respectively, against values close to 0% in the control. In stages 1, the cytotoxicity range was 4.0-50% for inducted cells and 1.0-4.0% for control cells.

Due to the unexpected small differences and even negative values resultant from a high signal for the background (absorbance values for culture medium alone), the culture medium was changed to α MEM, which does not have phenol red (stage 3 of the optimization process). Although producing a considerably lower background signal, the values were still high and when calculating the cytotoxicity for the control groups, they were high enough to produce negative values for this parameter, which is not reasonable. By this time, the mistake concerning the concentrations of H_2O_2 was discovered and the correct value was used in stage 4. In this stage, since the cells did not die in such a massive way, it was possible to count the viable cells and normalize the values for the cytotoxicity. Hereinafter, the percentage of cytotoxicity for the control group was considered to be 0%.

With the cell count enabled in stage 4, other clear issue of this assay arose: the different cell growth rates between control and inducted wells. With the addition of H_2O_2 to the culture medium, a decrease in the number of cells, comparing to the number in the control group, was expected. In some cases, however, the inducted groups had a higher number of cells than the control groups, which pointed out that probably, during the culture time, the cells had a greater proliferation in the wells that were later subjected to the induction. Therefore, when calculating the number of dead cells, this value became negative and, consequently, the cytotoxicity was also negative. This could not be possible since a treatment with an agent that was already proved to be very aggressive to the cells could not stimulate the cells to proliferate in a higher rate than the death rate it should induce. To overcome this difference in the cell growth rate, the cells were not allowed to proliferate for so such a long period. Therefore, the cells were seeded at a higher initial cell-density (to provide enough cells for flow cytometry later on), and the culture time was reduced from 4 days to only 1 (stage 5).

Despite this adjustment at stage 5, there was still one case of a negative value of cytotoxicity due to discrepancies in the number of dead cells. Besides, the medium controls continued to show high values of background and so, the positive values for the cytotoxicity seemed to be reduced given the treatment the cells had received, varying only from 2.0-5.0%. The results from FITC Annexin V staining were also corroborating the absence of oxidative stress between control and inducted cells. The results obtained in stages 4 and 5 seemed to point to a reduced effect of 100 μM of H_2O_2. This led to stages 6 and 7, where the concentration of H_2O_2 was doubled, the time of incubation reduced to 6 hours and the percentage of FBS tested in an attempt to reduce the background signal: 10% in stage 6 and 5% in stage 7.

The increase in the concentration of H_2O_2 did not seem to stimulate a higher cell death, neither in the LDH measurements nor in the flow cytometry results. Furthermore, the reduction of FBS conducted to a slight increase in the background signal, instead of decreasing it. This could possibly result from a short incubation time with the oxidant agent and so, stages 8 and 9 took place, given that

in both, the initial concentration of H_2O_2 was used. The differences resided in the percentage of FBS and the time of incubation (5% and 24 hours for stage 8, and 10% and 48 hours for stage 9, respectively). While stage 8 settings were unsuccessful in producing a reliable value for the cytotoxicity of inducted groups, the settings of stage 9 seemed to have given rise to the best results so far, with a cytotoxicity of 10% and higher values of early and late apoptosis rates when compared to the control group as shown in the flow cytometry results. However, such a long period of incubation could bias the results since the calculation of the number of dead cells would be influenced by the proliferation of the cells in the control group, thus not reflecting so faithfully the effect of the oxidative stress on the viability of the cells.

In 2012, the group of Ertas studied the resistance of human BM-MSCs and ADSCs to an oxidative stress induced in cells seeded in 6-well plate wells, at a density of 10 x 10^4 cells/cm^2, by 2 mM of H_2O_2 incubated for 1 hour[320]. Using the same Annexin V FITC kit, they observed that ADSCs were more tolerant to the toxicity induced by the oxidant agent, presenting a late apoptotic rate of 10.29%, against 20.77% by BM-MSCs[320]. With this study, the authors were capable of not only differentiate the oxidative stress resistance potential of each type of cell but also show that, at least, the staining with Annexin could provide results using a short incubation time, although with a concentration of H_2O_2 10-fold higher than the last value used herein, and a much higher initial cell density. Nevertheless, their findings of an improved performance for ADSCs was in opposition to what Peng and co-workers (2008) had seen previously, after an incubation of 90 minutes with the same concentration of H_2O_2[193]. It is noteworthy that both groups found that ADSCs were the most resistant to serum-deprivation induced apoptosis[193; 320]. Also in 2012, the group of Li did not observe a statistically significant difference in the rate of apoptosis of ADSCs and BM-MSCs using the Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) assay[336]. In that assay, they used only 100 μM of H_2O_2 incubated for 24 hours, although they could not reach a statistically significant higher apoptosis rate in the induced groups compared to the respective controls[336].

IV.3 Isolation, Expansion and Immunophenotypical Characterization of Mesenchymal Stem/Stromal Cells from Adipose Tissue

IV.3.1 Stromal Vascular Fraction Isolation

The standard processing method of AT is based on enzymatic processes[184] that show some disadvantages, namely due to the use of enzymes, like collagenase, dispase and hyaluronidase, which are of animal or bacterial origin, expensive products especially if they are clinical grade[321; 348] and also because these products show lot-to-lot variability and are considered to introduce more than minimal manipulation in the processed MSCs[321]. Hence, the conceiving of a processing method free of enzymes would be desirable. In 2013, Shah and colleagues proposed a non-enzymatic method where mechanical forces play the major role in the isolation of the target cell population[321].

Regardless of the method that is used to process the AT sample, the resulting cell population is the so-called SVF[194].

In this work, both enzymatic and non-enzymatic processing methods (described in section III.8) were used in order to isolate AT derived SVF and ADSCs. Figure IV-5 shows the total cell number (SVF) that was obtained using each processing method.

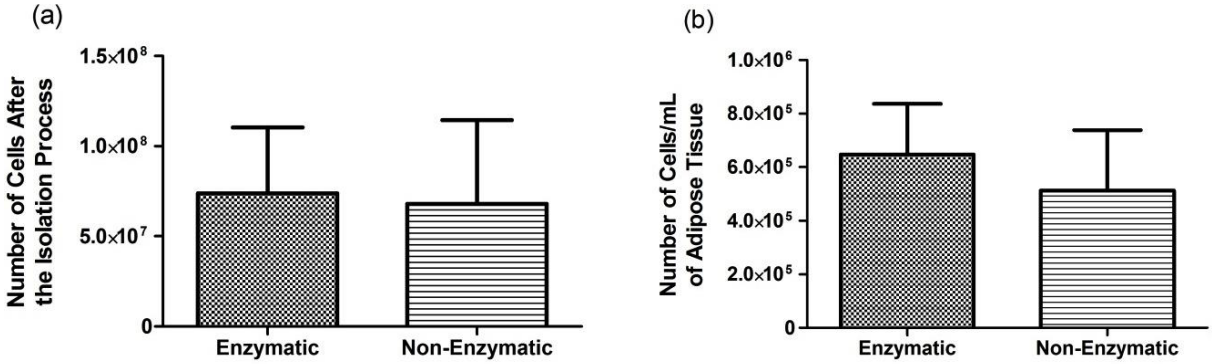


Figure IV-5. Isolation of SVF from AT samples by enzymatic and non-enzymatic methods. (a) Total cell number after the isolation process; (b) Number of cells/mL of adipose tissue. For both graphics, n = 11 and n = 9 for enzymatic and non-enzymatic methods, respectively. Results are presented as mean ± SEM.

All the samples of AT indicated in VII.3 were processed using both methods except for L140325, since the cell counts after the non-enzymatic processing were null (and so only the enzymatic method was considered), and L140627, that due to small sample volume (50 mL) was only processed by the enzymatic method.

The mean number of SVF cells (Figure IV-5 a), obtained in the enzymatic and non-enzymatic method was 7.4 ± 3.7x10^7 and 6.8 ± 4.7x10^7, respectively. These values were not significantly different ($p = 0.9196$). In order to compare the isolation yield obtained in this work with the yields referred in the literature, the values were normalized with the volume of AT sample (in mL) from which the cells were extracted. The resultant values for the enzymatic and non-enzymatic method were 6.5 ± 1.9x10^5 cells/mL and 5.1 ± 2.3x10^5 cells/mL (Figure IV-5 b), respectively and, once again, the difference between them was not significant ($p = 0.6509$).

In 2006, Yoshimura and co-workers described a non-enzymatic processing method of AT, similar to the one of Shah and colleagues (2013), that was the starting point for the method created by the latter group, where the SVF was obtained from the liposuction aspirate fluid of the AT samples, without further washes of the fatty portion[349]. In their study, the cell yields were 1.3 ± 0.5x10^6 and 1.6 ± 0.8x10^6 cells/mL, respectively for the enzymatic and non-enzymatic methods, with a non-statistically significant difference ($p = 0.401$)[349]. Comparing the results obtained in the present work with the ones from Yoshimura and co-workers (2006), it is observable that the latter ones are considerably higher. This might be partially explained by the cell counting method that was used by the group of Yoshimura (2006). The group used NucleoCounter (Chemometec, Allerod, Denmark), which is reported to be a fast, precise and objective tool for cell counting, and so it is a more reproducible method than the conventional counting methods[350]. Herein, the cell counting was performed manually using Turk's Reagent Solution, as mentioned in section III.8. When under the optical microscope, the SVF cells were very small, interspersed by several focus planes, and frequently hard to distinguish from waste particles. Additionally, in the present work, the AT samples were processed between 1-5 days after the harvesting. Such long periods from the harvesting to the processing might have reduced the

number of viable cells at the end of the SVF isolation. Despite the lack of information about the elapsed time between the referred processes in the study by the group of Yoshimura (2006)[349], the excessive period observed herein may account for the lower yield of cells when compared to the mentioned study.

All of the aforementioned factors might have contributed to the differences in the number of SVF obtained for each sample when compared to the literature. However, it is noteworthy that the yields of the two methods are quite similar, both herein as in the referred study. In future work, the yield of SVF cells may be increased by performing the AT processing as soon as possible, ideally within the 24 hours after the harvesting, to avoid the loss of ADSCs[182].

Another aspect that should be noticed when comparing both isolation methods is the processing time of each one. The enzymatic process took about 1 hour and 25 minutes to be concluded, while the non-enzymatic required less than half of that time, only 35 minutes. Comparing to the tissue processing times reported by Shah and colleagues (2013) (3 hours for the enzymatic and 1 hour for the non-enzymatic[321]), the processes performed in this work were faster, with maintenance of the tendency of the non-enzymatic method being a faster process than the enzymatic method. In the case of the enzymatic method, the time difference might be explained by the shorter incubation time of the AT samples with the collagenase solution, only 30 minutes in the present work against 1 hour in the work of Shah and colleagues, 2013[321]. In fact, the AT processing by an enzymatic method has been performed in the SCBL-RM and after an optimization of the process, 30 minutes were found to be sufficient to generate a SVF. Regarding the non-enzymatic method, the volumes of AT processed were reduced in this work (mean of 100 ± 20.2 mL), while Shah and co-workers (2013) had more variable volumes, between 60-300 mL[321]. Smaller volumes of AT take less time in the washing steps due to a faster separation of the tissue supernatant from the aqueous infra-natant.

IV.3.2 Adipose-derived Stem/Stromal Cell Isolation

To obtain the population of ADSCs, the SVF was platted onto T-flasks and cultured until it reached 70-80% of confluence, using two different culture media, DMEM+10% FBS or StemPro® MSC SFM (from now on mentioned as DMEM and XSF culture medium, respectively), as described in section III.8IV.3.2. The results of the ADSCs isolation through plastic adherence using both types of processing methods and culture media are shown in Table IV-4.

The rationale behind this comparison is the disadvantages of using a culture medium with poorly defined, animal origin components, such as FBS. Although it is considered a supportive component for the growth and attachment of cells, FBS should not be used in the context of a clinical cell therapy since it might induce immune responses when in contact with the human immune system[351] due to the possible presence of bacteria, virus, or xenogeneic antibodies[352]. Besides this, FBS presents some variability amongst lots, which might then translate into alterations of the proliferation rate or differentiation potential of stem cells[351]. Ergo, there is an interest in developing a fully defined xeno- and serum-free medium to culture cells and allow their use in the clinical context, skipping the issues of the actual standard media supplemented with animal or human components. StemPro® MSC SFM, a commercially available formulation (Gibco®) was used with such purpose.

Table IV-4. Isolation of ADSCs using enzymatic or non-enzymatic methods and using DMEM+10% FBS or StemPro® MSC SFM as culture media.

Conditions		Isolation success rate	Number of cells obtained (P0)/mL of Aspirate (mean ± SEM)
DMEM+10% FBS	**Enzymatic**	100% (7/7)	$2.6x10^5 \pm 1.5x10^5$
	Non-Enzymatic	50% (2/4)	$4.5x10^4 \pm 0.2x10^4$
StemPro® MSC SFM	**Enzymatic**	62.5% (5/8)	$4.9x10^5 \pm 3.5x10^5$
	Non-Enzymatic	0% (0/3)	-

For the DMEM culture medium, ADSCs were successfully isolated from only half of the samples processed by the non-enzymatic method, while the isolation success rate was 100% for the enzymatic method (Table IV-4). Using XSF culture medium, it was not possible to isolate ADSCs through the non-enzymatic processing method, while the cells from more than half of the samples processed by the enzymatic method successfully adhered to the flasks, proliferated and gave rise to an ADSCs population.

In the first 24 hours after the initial cell plating there were many cell contaminants in suspension, mainly red blood cells. Furthermore, this contamination appeared to be higher in the cells isolated through the non-enzymatic method.

The isolation rate reported in this work for the XSF culture medium using the enzymatic method is in accordance with the literature. In 2013, Patrikoski and colleagues reported an isolation success rate of 66.7%[351] which is similar to the one obtained in this work (62.5%). The low adhesion efficiency may have led to the complete failure of ADSCs isolation by the non-enzymatic method herein observed.

The average number of ADSCs isolated upon P0 by the enzymatic method was superior ($2.6 \pm 1.5x10^5$) to that by the non-enzymatic method ($4.5 \pm 0.2x10^4$) in DMEM, although it was not statistically significant ($p = 0.5983$). Nonetheless, the enzymatic method had a yield 5.7-fold superior to that of non-enzymatic method. Shah and colleagues (2013) reported similar values relatively to the order of magnitude, respectively $4.8x10^5$ cells/mL and $2.5x10^4$ cells/mL for the yield of enzymatic and non-enzymatic methods[321]. The authors found, however, that the enzymatic method was 19-fold more efficient than the non-enzymatic[321]. Herein, a very high initial density of cells from the SVF was generally used for the initial plating, 100 000 cells/cm^2 [192; 195]. The initial density used by the group of Shah was not specified[321], however differences in this parameter might explain the disparities between the values found in this work and those stated by Shah and colleagues (2013). It is noteworthy that the enzymatic method is, both herein and in the literature[321], more efficient than the non-enzymatic method when using FBS-supplemented DMEM. A possible explanation for the lower ADSCs yield in the non-enzymatic method is provided in section IV.3.4.

The yield of ADSCs isolated by the enzymatic method and the XSF culture medium was superior to the yield for the same method using DMEM, $4.9 \pm 3.5x10^5$ and $2.6 \pm 1.5x10^5$, respectively, even though the difference was not statistically significant ($p = 0.5983$). This tendency of having a higher yield for the XSF culture medium complies with the results obtained by Patrikoski and co-workers (2013), who found a higher proliferation rate of ADSCs cultured in this culture medium[351]. In

fact, during the culture of the cells in XSF culture medium, an obvious difference in size and border definition was noticed, with the cells being smaller and sharper than the cells cultured in DMEM, which were bigger and had a rougher delimitation. Patrikoski and co-workers (2013) also observed this distinctive shape amongst cultures and attributed such differences to the low initial adhesion of the cells in the XSF culture medium (equal to the one used herein)[351]. Since both cell cultures were harvested approximately at the same confluence stage, 70-80%, a smaller cell size leads to a higher number of cells covering the same area which thus translates into a higher number of cells obtained at the end of the isolation process for the XSF culture medium.

IV.3.3 Proliferative Potential Evaluation of Adipose-derived Stem/Stromal Cells

After the isolation of the ADSCs the proliferative potential was evaluated for each sample. When the immunophenotype of these cells was confirmed, the sample was cryopreserved and the proliferative study was finished. Thus, ADSCs isolated either by the enzymatic method or by the non-enzymatic method, using DMEM or XSF culture medium, were expanded between P1 to P4 and, at each passage, the following parameters were estimated (as described in section III.4): (1) cell density, (2) FI, (3) CFI, (4) PD and (5) CPD. For the isolation passage, P0, only cell density was also calculated but not the remaining parameters since the initial density of ADSCs was unknown. Such parameters are important in order to determine if a certain isolation method, associated to a specific culture medium, provides the necessary conditions for the cells to be expanded into clinically meaningful numbers that allow a cellular therapy. The results for the mentioned parameters are displayed at Figure IV-6.

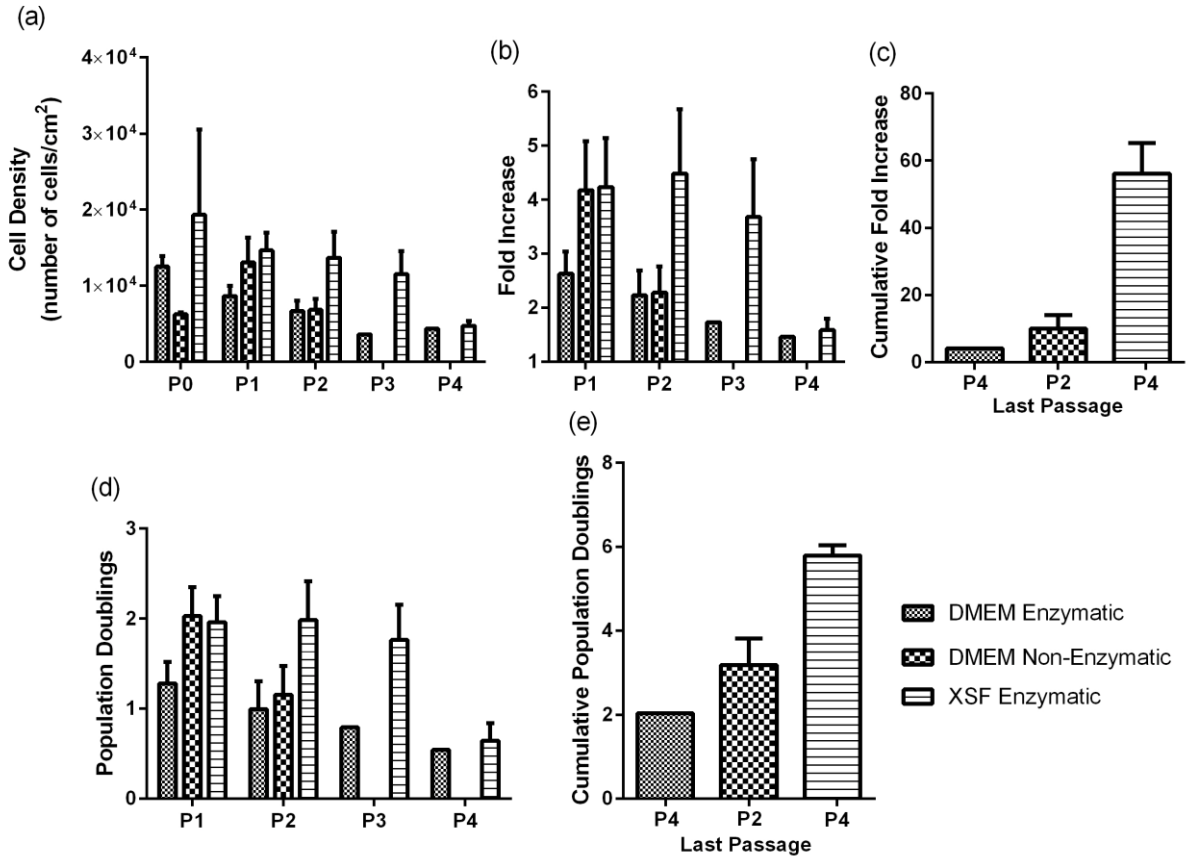


Figure IV-6. Evaluation of the proliferative potential of ADSCs isolated by the enzymatic or the non-enzymatic methods, and using DMEM+10% FBS or StemPro® MSC SFM as culture media. (a) Cell Density (number of cells/cm^2); (b) Fold Increase; (c) Cumulative Fold Increase; (d) Population Doublings; (e) Cumulative Population Doublings. The number of samples for each graphic is the following, (each cluster contains the number for each passage and the clusters are ordered by DMEM Enzymatic, DMEM Non-Enzymatic, and XSF Enzymatic): (a) n = (7, 7, 6, 1, 1), (2, 2, 2, 0, 0), (5, 5, 4, 3, 2); (b) and (d) n = (7, 6, 1, 1), (2, 2, 0, 0), (5, 4, 3, 2). For (c) and (e), n = 1 (P4), 2 (P2), and 2 (P4) for cells in DMEM Enzymatic, DMEM Non-Enzymatic, and XSF Enzymatic conditions, respectively. Results are presented as mean ± SEM.

Figure IV-6 shows a trend of ADSCs to have a higher proliferative potential when cells are isolated using the enzymatic method and XSF culture medium. Considering only the data for P0 and P1, ADSCs obtained by the enzymatic method and expanded in XSF culture medium consistently had higher cell densities, 1.9 ± 1.1x10^4 and 1.5 ± 0.2x10^4 cells/cm^2, than the cells expanded in the FBS supplemented culture medium, which achieved cell densities of 1.3 ± 0.1x10^4 and 8.6 ± 1.3x10^4 cells/cm^2 in the enzymatic method, and 6.3 ± 0.3x10^3 and 1.3 ± 0.3x10^4 cells/cm^2 in the non-enzymatic method. These slight differences could not be analysed by a two-way ANOVA since the number of samples was not the same neither for the isolation/expansion conditions nor for the passages. Nevertheless, a higher proliferative rate for the XSF conditions was also observed by the group of Patrioski (2013), which shows the harmony between the present findings and those of the literature[351]. Comparing the enzymatic and non-enzymatic methods, the first resulted in a higher density of cells at P0, as it was already stated in IV.3.2. However, it appears that ADSCs from the non-enzymatic method were capable of recovering from their isolation process and surpass the performance of the cells from the enzymatic method, which is further supported by a higher FI and PD at P1, although these displayed a more delayed proliferation. In fact, ADSCs from the non-enzymatic method took

about 19 days to expand from P0 until P1, while the cells from the enzymatic method took only 12 days, considering the DMEM medium. The more time-consuming expansion of ADSCs by the non-enzymatic method is confirmed by the studies of Shah and colleagues (2013)[321] and Yoshimura and colleagues (2006)[349], although there is no mention concerning the FI or the PD at each passage, which hinders a direct comparison of those parameters with the results obtained herein. It is noteworthy that although these cells take longer time to proliferate, this is not an obstacle for them to reach higher values at the end of the cell passage. However, caution must be taken when interpreting these results since the number of samples for the comparison is neither the same for both conditions nor sufficient to be statistically significant.

The low number of samples already mentioned in this proliferative study may have biased the results, especially for passages 2-4, for ADSCs expanded in DMEM. In fact, there was a reduction of the FI and PD from P2 to P3, in parallel to a decrease from 6 samples to merely 1, in the case of ADSCs from the enzymatic method. For the non-enzymatic method, there is no data after P2. Thus, any assumptions for these conditions at those passages must be done with caution. For ADSCs expanded in XSF conditions, there might also be a bias of the results at higher passages, where the number of samples is also smaller. Notwithstanding, the CFI and the CPD for these cells was, respectively, 30 ± 2.1 and 5 ± 0.1 at P3, and 60 ± 9.2 and 6 ± 0.20 at P4. For ADSCs expanded in DMEM and isolated by the enzymatic method, the CFI and the CPD were, respectively, 2.8 and 1.5 at P3, and 4.1 and 2.0 at P4. The values of the CPD at P3 and P4 for the cells from these two conditions are in agreement with those found by the group of Lindroos (2009), although their starting passage was not P0 but P3[353]. In fact, for DMEM and XSF culture medium, the authors obtained, respectively, 1-2 and 4-5 at P3, and 2 and 6 at P4[353].

IV.3.4 Immunophenotypical Characterization of Adipose-derived Stem/Stromal Cells

After isolating the ADSCs population for each sample, and in the lowest passage possible (from P0 to P3), the expression of a set of surface markers for MSCs was analysed by flow cytometry to certify the identity of the cell population obtained (as described in section III.9.1). Such set comprises some of the markers advised by the Mesenchymal and Tissue Stem Cell Committee of the ISCT[180] and is specified in Table VII-2 from Annexes. This immunophenotypical characterization is further crucial to highlight similarities and differences of ADSCs regarding their isolation method, enzymatic or non-enzymatic, and the culture medium used for isolation and expansion, DMEM or XSF. The results are displayed in Figure IV-7.

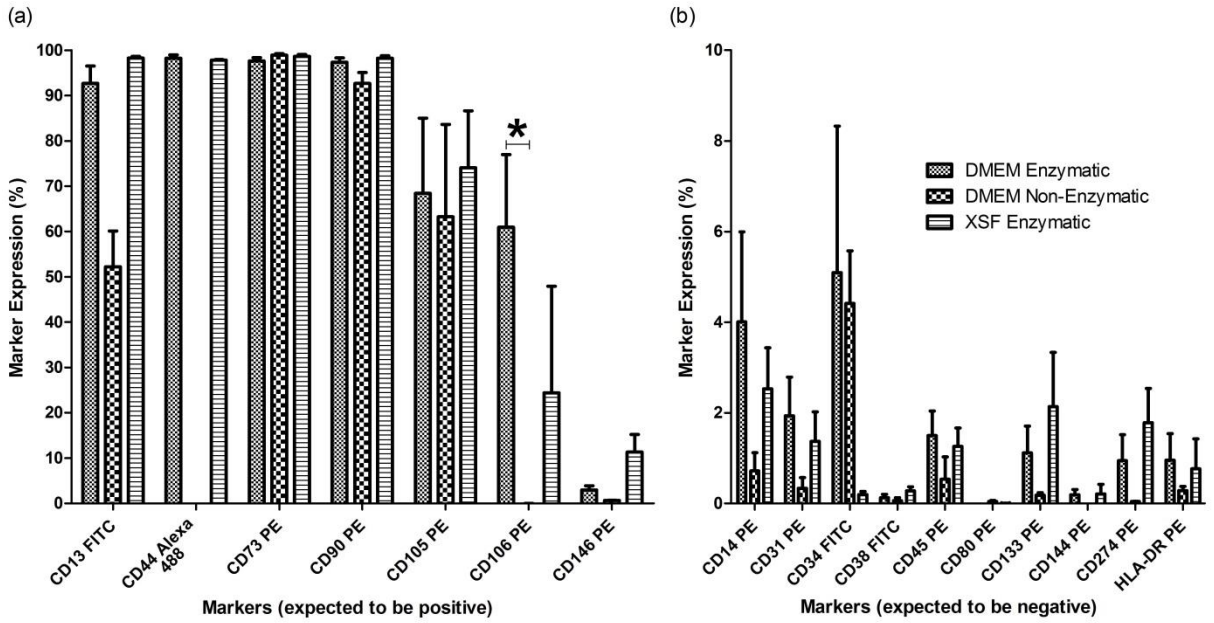


Figure IV-7. Immunophenotypical analysis of ADSCs successfully isolated. (a) Expression of markers expected to be positive for MSCs; (b) Expression of markers expected to be negative for MSC. In both graphics, n = 7, n = 2, and n = 4 for DMEM enzymatic, DMEM non-enzymatic, and XSF enzymatic methods, respectively. The only exception occurs for CD44 marker, in which case n = 6, n = 0, and n = 4. The samples analyzed were from P0-P3. Results are presented as mean ± SEM. * p < 0.05.

Looking at the overall picture, some of the positive markers of MSCs had an expression higher than 95%, as advised by the Mesenchymal and Tissue Stem Cell Committee of the ISCT[180], namely CD44 (for which it was not possible to perform the analysis in the cells isolated by the non-enzymatic method) and CD73. CD44 is an adhesion molecule involved in cell-to-cell and cell-to-matrix interactions, with the latter having hyaluronic acid as the main ligand, and is present mainly in cells of lymphohematopoietic origin[354]. Much lower values were reported by other studies for the expression of CD44 by cells from the enzymatic method and expanded in DMEM, approximately 13-66%[321; 353], while the values for ADSCs expanded in XSF culture medium (98 ± 0.1%) seem to be in agreement with the literature (98.1 ± 2.8%)[353]. However, the differences found by the group of Lindroos (2009) were not statistically significant[353]. The high expression levels of CD73 for cells expanded in both types of culture medium is supported by Patrikoski and colleagues (2013)[351], while Shah and co-workers (2013) found significant differences between cells from enzymatic and non-enzymatic method, with an expression of approximately 89% and 100%, respectively[321].

In the case of CD90, its expression was slightly lower for cells isolated by the non-enzymatic method, although the expression was still above 90% (93 ± 2.4%), while the expression was equally high in cells expanded in both types of culture medium, varying between 97 ± 1.0% and 98 ± 0.5%. Although the group of Lindroos (2009) could not obtain so high values for this marker on their study, they did not find significant differences for the latter observation[353]. Contrary to the observations in this work, Shah and colleagues (2013) found higher expression levels of CD90 for the cells isolated by the non-enzymatic method[321]. CD13 had an expression higher than 90% and similar for both enzymatic method groups (93 ± 3.8% in DMEM and 98 ± 0.3% in XSF culture medium), as found by Lindroos and colleagues (2009)[353], but for the non-enzymatic group, its expression fell to 52 ± 7.9%. Although

lacking a statistical significance, possibly due to a low number of samples in the non-enzymatic group, it is reasonable to accept a lower expression of this marker in cells isolated by this method. In fact, CD13 is associated to the adhesion capacity of MSCs and a reduced expression of this marker impairs the adhesion of MSCs and, consequently, the engraftment of the cells into locals of injured muscle[355]. Since cells isolated by the non-enzymatic method are found in the fluid portion of the liposuction aspirates, such location might indicate that their adhesion process is not so efficient, therefore highlighting an interference in the expression of CD13. The possibly lower adhesion efficiency of cells isolated in these conditions (non-enzymatic method and DMEM) may also account for the lower cell yielding obtained for the isolation of ADSCs at P0, referred in IV.3.2. The expression of CD105 was varied, approximately 63 ± 20.4% to 74 ± 12.6%, being higher for cells expanded in the XSF culture medium and lower for cells isolated by the non-enzymatic method, without statistical significance. Despite the reported high expression of CD105 in Shah's (2013) and Patrikoski's (2013) studies, superior to 95%[321; 351], and slightly more modest in the study of Lindroos and colleagues (2009)[353], a recent report from Mark and colleagues (2013) found a profound reduction of the expression of this marker in XSF conditions when compared to media supplemented with FBS, respectively 52 ± 15% and 96 ± 2.4%[356]. The dissimilarities found across these studies suggest the need to further evaluate the expression of this marker by ADSCs using a larger number of samples to reach statistically significant differences between conditions.

CD106 had an almost null expression for the cells isolated by the non-enzymatic method (0.02 ± 0.02%), reaching 61 ± 16.0% for the cells expanded in DMEM and isolated by the enzymatic method and 24 ± 23.5% for the cells expanded in the XSF culture medium. The difference between the CD106 expression in cells from enzymatic and non-enzymatic methods isolated in DMEM was the only with statistically significance reported amongst all the markers analysed ($p < 0.05$). The expression of this marker has been reported as being low for ADSCs, independently of the culture medium used[353; 357]. In fact, the expression of CD106 (important for the haematopoietic process) is high for MSCs derived from the BM since it is in this location that haematopoiesis occurs[357]. In a study from 2010, Han and colleagues proved the existence of HSCs in AT, albeit in a much lower frequency than in the BM, approximately 14-fold less[358]. Therefore, the levels of expression of CD106 observed herein for ADSCs isolated by the enzymatic methods are not so unreasonable. The expression of CD146 was overall low, being highest for cells expanded in XSF culture medium, reaching almost 11% of expression but without statistical significance. The low expression of this marker is also supported by the group of Lindroos (2009), who reported an expression inferior to 2% for cells expanded in both types of media[353].

The expression of CD31, CD45, CD144, and HLA-DR was under 2% as recommended[180]. CD133 had an expression below 2% for the cells expanded in DMEM, and a little more increased for the ADSCs expanded in XSF culture medium, albeit inferior to 5%. The negative expression of these markers is supported by other studies for the conditions analysed herein[321; 349; 351; 353]. Although without a statistical significance in this work, the expression of CD45 (hematopoietic lineage) is slightly lower for the non-enzymatic method, as the group of Shah (2013) observed[321]. Other markers also showed to be negative, like CD38, CD80, and CD274. Despite the slightly increased expression for CD80 in

the XSF conditions found in the study of Patrikoski and co-workers (2013), between 2% and 7%[351], this marker had an expression close to 0% in both types of culture medium. In the case of CD14, the expression was below 2% only for the cells from the non-enzymatic method, although it remained under 5% for the cells isolated by the enzymatic method and from both culture media. The low expression of CD14 was also observed by the group of Patrikoski (2013) for cells expanded in both DMEM and XSF culture medium[351]. The slight increased expression of CD14 in the cells isolated by the enzymatic method might point to a small contamination with monocytes, which express this marker[359].

The "negative marker" with more controversial results when it comes to compare the experimental results herein obtained and those presented in the literature is CD34. This marker presented a decreased expression for cells expanded in XSF culture medium, inferior to 1%, and an expression somewhat superior for cells expanded in DMEM, between 4 ± 1.2% and 5 ± 3.2%. This tendency seems to be in agreement with the study of Lindroos and co-workers (2009)[353], albeit an opposite trend was observed by the group of Patrikoski (2013), with the expression of CD34 varying from 7% to 41% for the first type of cells[351]. A significant difference of CD34 expression was also described by Shah and colleagues (2013), being lower for cells isolated by the non-enzymatic method, around 10.7 ± 9.2%, and much higher for cells isolated by the enzymatic method, ascending to 65.3 ± 36.3%[321]. In this latter study, the high expression of CD34 indicates a significant contamination of the target cell population by hematopoietic progenitors and endothelial cells, although their values were related to P0[321], while the ones presented herein vary from P0 to P4 and decreased throughout the passages. The disparity in the passages used to analyse the cells may, therefore, account for the differences observed when comparing to both Shah' (2013) and Patrikoski' (2013) studies[321; 351].

V. Conclusions and Future Trends

The clinical application of MSCs has been studied throughout the past years in diverse areas, from immunity dysregulations[219; 223; 249] to co-transplantation with HSCs[253] and regeneration of several tissues[255], including the myocardium. In fact, the cardiac regeneration has been raising a lot of interest in the research community since the discovery that the human heart, unlike what was previously thought, has a potential to regenerate itself[118; 119], albeit at an insufficient rate to induce a full recovery following an AMI[119]. These findings, allied to the fact that CVDs are the leading cause of death worldwide[34] and their burden is expected to increase in the near future[6], has driven the search for the development of a therapy that, unlike the conventional ones, would be capable of a truly regeneration of the heart[119]. Due to their intrinsic features, MSCs appear to be a great candidate for the development of an efficient therapy, although the mechanisms by which they contribute to the improvement of the heart condition still motivates discussion amongst experts[121; 242; 256-258; 264; 265; 267]. Indeed, the unique properties of MSCs, namely the immunomodulation[218], hypoimmunogenecity[233] and regenerative properties[242; 243; 246] allow their use in either an autologous or allogeneic settings[279]. Aiming at the comparison of the therapeutic potential of these cells in each setting, this work focused on the evaluation of key properties that might be the most useful in the clinical setting, namely the capacity of being expanded *ex-vivo* to overcome the limited *in vivo* cell numbers, the secretory profile, the angiogenic potential and the resistance to oxidative stress. These properties were investigated for MSCs extracted from BM or AT of healthy donors and for BM-MSCs from AMI patients, expanded in normoxia or hypoxia, to test the positive effect of hypoxic conditions in the proliferative potential, both in the cell yield and the reduction of expansion time, as previously reported[328]. Two different methods for the isolation of ADSCs from AT samples were further explored in order to confirm the AT as a reliable source of MSCs.

In the proliferative study of BM-MSCs, from both healthy and AMI patients, and ADSCs, the main results were in agreement with the literature. The CFI and CPD obtained for the three types of cells evaluated in this work showed a consistent higher performance of the cells expanded under hypoxic conditions[329-332]. In fact, despite lacking statistical significance, AMI BM-MSCs achieved superior values at P9 in comparison to every other cell type, except for hypoxic ADSCs. For normoxia conditions, AMI BM-MSCs did not present an improved performance than healthy BM-MSCs and ADSCs expanded in the same oxygen tensions. The main reasons for this outstanding performance of AMI cells in the hypoxic conditions might be the younger age of the majority of the donors in this group, as well as the previous exposure of the cells to the inflammatory response, activated after the ischemic episode of the AMI, in which some cytokines, like TNF-α, stimulate the proliferation of BM-MSCs, as observed by Prasanna and colleagues (2010)[335]. ADSCs were also observed to provide higher cumulative values of the referred parameters, when compared to healthy BM-MSCs, but only under hypoxic conditions. This higher proliferative rate was further supported by several other studies[193; 195; 200]. The results obtained in this proliferative study could gain more consistency and suffer less impact from operator errors if performed in fixed time points and using a higher number of samples in the three groups of cells.

The analysis of the secretory profiles of MSCs as a response to a stimulated inflammatory scenario, in normoxia and hypoxia conditions, showed that, in fact, pro-inflammatory cytokines like TNF-α and IFN-γ enhanced the production of VEGF and IL-6 in a statistically significant manner for the latter cytokine. This effect was supported by other studies[230; 317; 337]. The impact of hypoxia conditions also stimulated the increase of VEGF levels for the three types of cells, as reported by the groups of Kinnaird (2004)[268] and Rehman (2004)[243], although the same was not observed for the production of IL-6. The impact of the source of the cells in the expression of IL-6 upon stimulation seemed to be more controversial, since herein, no statistical differences existed amongst cell types, although, in fact, higher values of VEGF were associated to healthy BM-MSCs, as seen by Li and colleagues (2012)[336]. The basal levels of VEGF were similar for both the BM-MSCs, regardless the oxygen tension, and both were higher than those for ADSCs. Nevertheless, upon stimulation, the concentration of VEGF was similar for ADSCs and AMI BM-MSCs. The several variations in the protocol for the induction with TNF-α and IFN-γ, namely for the cell-density upon seeding, the time for stimulation and the incubation time, hinder more sustained comparisons. It is also noteworthy that a normalization of the concentration levels obtained, for example through the number of cells present after the incubation time, could lighten the impact of some of those differences and eliminate the proliferation/cell number effect on the expression level observed for the different conditions. Therefore, a higher expression of a certain cytokine would be associated with a truly enhanced capacity of a single cell to secret higher levels of it, instead of being possibly linked to an increased number of cells expressing the protein.

It would be interesting to evaluate the secretory profile of these cells throughout their expansion process to estimate the impact of long *in vitro* expansion prior to the implantation of the cells into the patient.

The results obtained in the three settings for the angiogenic potential evaluation were quiet different amongst them. The angiogenic assay was not only capable of showing that MSCs are able to form tubular structures without the presence of cells possessing that intrinsic property, but also of differentiating the potential of each cell type, with the AMI BM-MSCs giving rise to a higher number of tubes than ADSCs which, in turn, presented more tubes than healthy BM-MSCs. Nevertheless, the latter cells had statistically significant longer tubes than controls and the other types of cells under normoxia, which might be associated to an increased secretion of VEGF, as seen in the secretory profile analysis. Overall, hypoxia conditions also induced the formation of slightly less but longer tubes, with a small impact in the number of connections, when compared to normoxia. The number of connections followed the tendency of the number of tubes.

To investigate if MSCs are really capable of acquiring a phenotype close to the one of ECs without their presence, the cells could be previously stained with DiI-Ac-LDL like they are in the co-culture system. In the case of incorporating the dye, the results would show that the tubular structures are somehow related to the ones created by ECs.

The use of conditioned media produced tubes visually more similar to the controls than any other setting, although in less quantity for ADSCs conditioned medium, smaller for both types of BM MSCs conditioned medium, and with an overall slightly reduced number of connections, which can be

seen by the presence of incomplete tubes in the correspondent images of Figure IV-3. The length of the tubes and the number of connections was, however, very similar amongst all the types of cells and oxygen tensions, pointing to the inefficacy of this assay to produce robust and distinctive results according to the type of cell involved. The assay also produced opposite results to the ones found in the literature, although this is possibly explained by the use of negative controls[343; 344], instead of positive as it was done herein. In the future, both controls should be performed to simplify the comparison of the results with the literature.

The tube incorporation assay produced results with a greater agreement to the ones reported in the literature. Once again, the apparent inversely proportion between the number of tubes and the respective length seemed to be present, with the co-cultures giving rise to considerably less but longer tubes when compared to control, and with statistical significance for the co-culture with ADSCs. The longer tubes formed by the co-culture of HUVEC and healthy BM-MSCs is further supported by the studies of Rahbarghazi and colleagues (2013)[344] and Duffy and colleagues (2009)[338], with the latter group confirming the formation of thicker tubes with larger junctions, as seen herein (Figure IV-3). Although being incapable of highlighting the effect of oxygen tensions, this setting showed that the presence of MSCs, instead of only their conditioned medium, may be important to induce the formation of more robust and complex tubular structures, possibly with an important effect of direct contact, beyond the paracrine effect[338]. To prove that the co-cultured tubes are, indeed, more robust than tubes formed by HUVEC alone, the network could be analysed throughout time, as the group of Duffy (2009) did[338]. The same could also be studied for the angiogenic assay.

Despite the long process of optimization of the oxidative stress resistance quantification assay, the results obtained were, most of the time, unreasonable, with negative values for the cytotoxicity and reduced differences between stimulated and non-stimulated cells for apoptotic rates and LDH levels being the main issues. In future studies, several values for the concentration of H_2O_2 should be tested, and higher initial cellular densities should also be considered, together with reduced times of incubation with the oxidant agent, similarly to the setting of Ertas and colleagues (2012)[320]. The results obtained with this assay would have been important to further consolidate the different performances of the three types of cells analysed and fundament the best choice for a clinical context.

In future work, the features of BM-MSCs harvested from the same AMI patients at different time points upon infarction should also be investigated and compared to cells obtained from healthy donors. Such study could clarify the source of the differences observed between AMI and healthy cells, as well as show if such differences are transitory or permanent.

Regarding the isolation methods of MSCs from AT (enzymatic and non-enzymatic), several conclusions might be withdrawn. While both yielded a comparable number of SVF cells as seen in the literature[349], the later one is simpler, faster, and skips products (such as collagenase solution) that could make it less defined, which makes it more economical and closer to generate a current good manufacturing practices product[348]. Nevertheless, the non-enzymatic method presented a non-statistically significant smaller yield of ADSCs at the end of the isolation process of this population, supported by Shah and colleagues (2013)[321]. This seems to be overcome during the proliferation,

where non-enzymatic isolated cells showed a higher, albeit slower[321], proliferative potential comparatively to cells isolated by the enzymatic method and expanded in DMEM.

In the future, the processing of AT should be performed within 24 hours and more information regarding the local from which the samples was extracted, the applied suction force, the cannula diameter, the type of processing, amongst others, should be collected since these parameters might affect the cell yield[182].

The XSF culture medium used herein demonstrated to be effective for the isolation of ADSCs, although it would be desirable to increase the initial adhesion of the SVF cells. Several initial densities of SVF cells should also be tested. These optimization steps could render some successful isolation cases for the non-enzymatic method using this culture medium. XSF medium was associated to an increased proliferation potential of ADSCs isolated by the enzymatic method, when compared to cells expanded in DMEM, similarly to the findings of Patrikoski and co-workers (2013)[351].

In general, the markers expected to be negative for MSCs presented a reduced expression in this work, consistent with the values reported in the literature[321; 349; 351; 353], and were similar amongst isolation methods and culture media. More controversial was the expression of the markers expected to be positive. The ones more concordant with the literature were CD13[353], CD73[351], and CD146[353]. CD106 was the only marker showing a statistically significant different pattern between isolation methods, being much higher for cells isolated by the enzymatic method than by the non-enzymatic. CD13 was less expressed by non-enzymatic isolated cells, which might be a consequence of the fact that the cells obtained by such method are in the fluid portion of the liposuction aspirates and thus might have an impaired adhesion capacity. The expression of this marker should be further investigated because in the case it is proven to be smaller for cells isolated with a non-enzymatic method, then the use of these cells in a clinical context might fail or be hampered by a decreased rate of cellular engraftment.

The dissimilarities observed between the experimental results obtained in this work and the values reported in the literature for the immunophenotypical characterization of the cells may be a consequence of the low number of samples analysed and mixture of analysis from different passages. Since time contributes to a homogenization of the ADSCs population[351], initial differences in the markers expression associated to the isolation methods and culture media might have diluted themselves throughout the experience, explaining, therefore, why some distinctive patterns observed in the literature are lacking in this work. However, ADSCs have a consistent immunophenotype, comparable to the expression reported in the literature for a great cluster of markers, regardless of the isolation method or the expansion culture media.

In the scope of this work, only ADSCs were subjected to an immunophenotypical characterization to confirm their identity. However, in future works, the same should be done to SVF cells to attest their heterogeneity. The identity of ADSCs should also be further confirmed through differentiation potential and clonogenic assays.

To conclude, the expansion of MSCs in hypoxia conditions should be considered, not only to provide higher PD but also to protect the cells from an excessive oxygen tension[332] and induce the

production of important cytokines in the context of an AMI, like IL-6 and VEGF. A pre-stimulation of the cells with inflammatory cytokines may be performed to further increase the secretion of those growth factors and maximize the paracrine action of the cells upon implantation into the infarct site. While healthy BM-MSCs displayed the best secretory and angiogenic potentials, AMI BM-MSCs had the highest proliferative capacity, followed by ADSCs, in hypoxic conditions. Furthermore, 2 out of 3 donors of the AMI group had less than 50 years, which corresponds to a younger age than the one usually seen in AMI patients[55] and might have induced the better proliferative potential seen herein. However, the comorbidities of the AMI donors are not known. Taking into account that age[280; 281] and comorbidities[282] have a negative impact in the features of MSCs and that the majority of the AMI patients are old[8] and have other diseases[55; 284-287], the autologous setting might not provide the best outcome for these patients. Instead, a cell bank of healthy donors with cells immediately available in relevant numbers and skipping the need for an immunocompatibility-matching is preferred. A higher number of samples in the three groups of cells studied herein should clarify the trends obtained in this work. ADSCs might be an acceptable alternative to BM-MSCs and it can be particularly advantageous if their *ex-vivo* expansion is performed under hypoxic conditions.

VI. References

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VII. Annexes

VII.1 Panel of Positive and Negative Markers for MSCs.

Table VII-1. Panel of markers that cells must or must not express to be classified as MSCs. The antigens marked (with a *) correspond to the cluster of markers referred by the Mesenchymal and Tissue Stem Cell Committee of ISCT as being part of the minimal criteria

Markers That Must Be Positive		
Marker	**Description**	**Reference**
CD13 (Aminopeptidase N)	Involved in the metabolism of biologically active peptides, growth and differentiation control, adhesion, cell-migration, angiogenesis, inflammatory trafficking, and phagocytes; Also present in monocytes, granulocytes, fibroblasts, and endothelium	181; 355
CD44 (Hyaluronic acid receptor)	Involved in homing; Also present in leukocytes, Kupffer's cells, fibroblasts, epidermal keratinocytes, and corneal cells	181; 354
CD73* (Lymphocyte-vascular adhesion protein 2)	Mediates activation of B cells and hematopoietic control; Also present in lymphoid tissue and endothelial lineage cells	180; 181; 209; 360
CD90* (Thy1 antigen)	Belongs to the family of immunoglobulins; Also present in T cells, granulocytes, early hematopoietic cells, epithelium, fibroblasts, and neurons	180; 181; 360
CD105* (Endoglin)	Transforming Growth Factor Beta receptor III; Also present in endothelium, syncytiotrophoblast, macrophages, and fibroblasts	180; 181; 360
CD106 (Vascular cell adhesion molecule-1)	Involved in immunosuppression and binding of hematopoietic progenitor cells; Also present in endothelial lineage cells	360-362
CD146 (Melanoma cell adhesion molecule)	Involved in cell adhesion protein in vascular endothelial cell activity and angiogenesis; Also present in endothelial lineage cells	360; 363; 364
Markers That Must Be Negative		
Marker	Description	Reference
CD11b* (Macrophage-1 antigen)	Mediates the multifaceted adherence reactivity of myeloid cells; Present in granulocytes and monocytes/macrophages coupled with CD18 in a heterodimer	180; 362; 365
CD14* (Myeloid cell-specific leucine-rich glycoprotein)	Enhances cell sensitivity to lipopolysaccharides; Present in all mature myeloid cells	180; 359; 362
CD19* (B-lymphocyte Surface Antigen B4)	Involved in B-cell signalling and/or growth regulation, and development of a humoral immune response; Present in B-cells	180; 362; 366
CD31 (Platelet Endothelial Cell Adhesion Molecule-1)	Promotes cell-cell adhesion and is involved in movement of neutrophils and macrophages across the vascular endothelium; Present in endothelial and hematopoietic cells	209; 362
CD34* (Hematopoietic Progenitor Cell Antigen CD34)	Primitive hematopoietic stem cell marker; Present in normal hematopoietic progenitors	180; 209; 367
CD38	Involved in activation and proliferation pathways; Present in activated T cells and B cells	368
CD45* (Leukocyte common antigen)	Present in hematopoietic cells	180; 362; 369
CD79α*	Is a part of the B cell receptor complex, necessary for antigen recognition and signal transduction in B cells; Present in B cells	180; 370
CD80	Involved in the activation of T cells	362; 371
CD133 (Prominin-1)	Involved in the hematopoietic reconstitution; Present in hematopoietic progenitors	362; 372
CD144 (Vascular endothelial Cadherin)	Involved in the endothelial cell contact integrity and in the control of the permeability of the blood vessel wall; present in endothelial cells	362; 372
CD274 (Programmed cell death 1)	Involved in the inhibition of immune responses; Present in natural killer cells, dendritic cells, activated monocytes, B cells and T cells	373
HLA-DR* (Human leukocyte antigen DR)	Involved in the immune response; Present in antigen presenting cells, namely dendritic cells, monocytes/macrophages, and B cells	180; 374; 375

VII.2 Panel of Markers for the Immunophenotypical Characterization of ADSCs.

Table VII-2. Panel of mouse anti-human monoclonal antibodies with the respective commercial brand, conjugated fluorophore and isotype, used in the immunophenotypical characterization of ADSCs.

Surface Marker	Commercial Brand	Conjugated Fluorophore	Isotype
IgG1/IgG1	BD Biosciences	PE/FITC	-
IgG1	BioLegend®	PE	-
IgG2b	BioLegend®	Alexa 488	-
IgG2a	BioLegend®	PE	-
CD13	abcam®	FITC	IgG1
CD14	BioLegend®	PE	IgG1
CD31	BioLegend®	PE	IgG1
CD34	BD Biosciences	FITC	IgG1
CD38	BioLegend®	FITC	IgG1
CD44	BioLegend®	Alexa 488	IgG2b
CD45	IQ Produtcs	PE	IgG1
CD73	BioLegend®	PE	IgG1
CD80	BioLegend®	PE	IgG1
CD90	BioLegend®	PE	IgG1
CD105	BD Biosciences	PE	IgG1
CD106	BioLegend®	PE	IgG1
CD133	Miltenyi Biotec	PE	IgG1
CD144	BioLegend®	PE	IgG2a
CD146	BioLegend®	PE	IgG2a
CD274	BD Biosciences	PE	IgG1
HLA-DR	BioLegend®	PE	IgG2a

VII.3 Donor Samples Data.

Table VII-3. Features of the BM and AT samples used in this study. * First value corresponds to the volume processed by the enzymatic method, while the second value corresponds to the volume processed by the non-enzymatic method.

Source	Donor Code	Gender	Year of Birth	Date of Harvesting	Date of Processing	Volume of Sample (mL, mL)*	Age	Age (Average ± SEM)
Healthy Bone Marrow	M48A08	Male	1948	2008	-	-	60	51 ± 8
	M50A08	Male	1950	2008	-	-	58	
	M72A07	Male	1972	2007	-	-	35	
AMI Bone Marrow	M55A11 AMI	Male	1955	2011	-	-	56	49 ± 4
	M62A11 AMI	Male	1962	2011	-	-	49	
	M70A11 AMI	Male	1970	2011	-	-	41	
Adipose Tissue	L090403	-	-	03-04-2009	-	-	-	-
	L090602	-	-	02-06-2009	-	-		
	L090706	-	-	06-07-2009	-	-		
Adipose Tissue Processed	L140318	Female	-	17-03-2014	18-03-2014	200, 200	-	41 ± 5
	L140325	Female	-	20-03-2014	25-03-2014	50, 50	52	
	L140326	Female	-	24-03-2014	26-03-2014	200, 200	38	
	L140331	Female	-	31-03-2014	03-04-2014	50, 50	52	
	L140403	Female	-	31-03-2014	03-04-2014	50, 25	28	
	L140404	Female	-	03-04-2014	04-04-2014	50, 50	36	
	L140411	Female	-	10-04-2014	11-04-2014	150, 125	26	
	L140414	Male	-	14-04-2014	16-04-2014	50, 150	43	
	L140416	Female	-	14-04-2014	16-04-2014	50, 75	74	
	L140513	Male	-	12-05-2014	13-05-2014	50, 100	30	
	L140627	Female	-	26-06-2014	27-06-2014	50, 0	30	