UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Scalable Platform for the Expansion of Human Mesenchymal Stem/Stromal Cells Under Dynamic Conditions

Francisco António Guilherme Moreira

Supervisor: Doctor Joaquim Manuel Sampaio Cabral

Co-supervisor: Doctor Cláudia Alexandra Martins Lobato da Silva

Thesis approved in public session to obtain the PhD Degree in

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Abstract

Mesenchymal stem/stromal cells (MSC) have emerged as a promising cellular product with huge potential for clinical applications. Due to their unique properties, these cells can be applied in the treatment of immune diseases, wound healing and even used for tissue engineering, among others. However, one of the major hurdles is that traditional culture systems are unable to meet the cell doses needed for clinical implementation. Beside bone marrow, the primary and most well studied source, umbilical cord and adipose tissues have been progressively approached with the objective of overcoming the drawbacks of invasiveness and low yield of MSC obtained through bone marrow. Nonetheless, the need for a reproducible and scalable platform, that allows ex-vivo expansion in a xeno(geneic)-free manner, remains indispensable.

The main objective of this thesis was to establish a three-dimensional dynamic culture that could be scaled into a fully controlled and automated platform, for the expansion of human MSC, in particularly adipose tissue-derived (AT) cells, under xeno-free conditions. To this end, two main approaches were followed: (i) MSC cultivated as aggregates, known as spheroids, that could undergo culture as a 3D structure in a scaffold-free environment, and (ii) MSC cultivated on microparticles placed in suspension, which offer surface area for adherent cells to expand, referred to as microcarriers.

Spheroids were found to be unable to support MSC expansion in both static and dynamic conditions tested. These 3D structures were characterized in regard to their assembly reproducibility, cell source and size. Different media, cell density and agitation were tuned with the objective of improving cellular proliferation, but with no success.

The need to establish a microcarrier-based platform for efficient expansion of AT MSC led to an initial screening of the available microcarriers. After careful selection of appropriate microcarrier, dynamic culture was optimized using a spinner flask platform. In comparison to StemPro®, a well-established xeno-free medium for human MSC, a human platelet lysate (hPL) supplement (UltraGRO™) and an in-house produced human AB serum (hAB) showed to improve culture conditions in regard to cell adhesion efficiency and maximal cell density attained. Coating strategies, feeding regiments and initial cell densities were optimized with the objective of translation to a higher scale bioreactor. Lastly, two automated and fully controlled bioreactors were used to implement the optimizations attained in the spinner flask system.

In conclusion, throughout this thesis it was emphasised the importance of a 3D dynamic culture for MSC expansion, having identified what steps must be taken to optimize and implement a scalable culture system capable of meeting the needs of the cellular therapy sector.

Keywords: mesenchymal stem/stromal cells (MSC); spheroids; microcarriers; human platelet lysate (hPL); human AB serum (hAB); Adipose Tissue

Resumo

Nas últimas décadas, as células estaminais/estromais do mesênquima (MSC) emergiram como um produto celular promissor devido ao seu potencial de aplicação clínica. Devido às suas propriedades únicas, estas células podem ser usadas para tratar doenças do foro imunológico, cicatrização de feridas, aplicações em engenharia de tecidos, entre outras. No entanto, um dos maiores desafios reside no facto dos sistemas de cultura tradicionais não conseguirem alcançar o número de células necessário para a sua implementação clínica. Para além da medula óssea, a primeira e mais bem estudada fonte celular de MSC, o cordão umbilical e tecido adiposo têm sido cada vez mais utilizados para ultrapassar as limitações de um método de colheita invasivo e o baixo rendimento na obtenção de MSC a partir da medula óssea. Não obstante, a necessidade de uma plataforma que seja reprodutível e facilmente escalável permanece um dos principais desafios.

O principal objectivo desta tese foi o estabelecimento de uma cultura dinâmica tridimensional que possa ser escavável para uma plataforma automatizada e totalmente controlada, que permita a expansão de MSC, em particular MSC derivadas de tecido adiposo (AT), sob condições sem componentes de origem xenogénica (xeno-free). Neste sentido, duas metodologias foram utilizadas: (i) cultura de MSC em agregados, chamados esferóides, cultivados como estruturas 3D, e (ii) cultura de MSC em micropartículas (*microcarriers*) que se mantêm em suspensão disponibilizando superfície de adesão para que células possam expandir.

Em cultura sob o formato esferóides, as MSC não proliferaram, tanto em condições estáticas como dinâmicas. Estas estruturas 3D foram caracterizadas quanto à sua capacidade de agregar, reprodutibilidade, fonte celular e tamanho. Foram testados diferentes meios de cultura, densidades celulares e agitações com o objectivo de beneficiar a expansão, mas sem sucesso.

Com o objectivo de estabelecer uma plataforma com *microcarriers* para a expansão eficiente de AT MSC, foi feita uma triagem em relação a diferentes *microcarriers*. Após essa selecção, optimizou-se a cultura dinâmica em spinner flask. A comparação entre StemPro®, um meio xeno-free já estabelecido, com meio suplementado com lisado plaquetário humano (hPL) ou soro AB humano (hAB), demonstrou que a utilização dos suplementos de origem humana promovia a adesão celular, assim como a densidade celular máxima. Foram também optimizadas estratégias de revestimento dos *microcarriers*, regimes de alimentação e inóculo com o objectivo de progredir para um bioreactor com automatação e controlo. Por fim, dois sistemas de bioreactor foram testados para implementar as optimizações feitas em *spinner flask*.

Em conclusão, esta tese procurou demonstrar a importância da implementação de uma cultura dinâmica 3D para a expansão de MSC e identificar quais os passos que podem ser seguidos para optimizar e implementar plataformas de cultura escaláveis capazes de corresponder às necessidades do sector das terapias celulares.

Palavras-chave: células estaminais/estromais do mesênquima (MSC); esferóides; microcarrier; lisado plaquetário humano (hPL); soro AB humano (hAB); tecido adiposo (AT)

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Dear all,

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I want to thank my supervisors for the opportunity of allowing me to pursuit this degree and for mentoring me in this amazing 5 years.

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Finally, a warm special thanks to my parents, brother and Ana “Elfa” for being all with me, no matter what. I love you all.

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Abbreviations

#
2-D – Two-dimensional
3-D – Three-dimensional
α-MEM – α-minimum essential medium

A
A/A – Antibiotic/Antimycotic
ASC - Adipose Stem Cell
AT – Adipose Tissue

B
bFGF – basic Fibroblast Growth Factor
BM – Bone Marrow
BSA – Bovine Serum Albumine

C
CB – Cord Blood
CM – Conditioned Medium
CXCR4 – C-X-C Chemokine Receptor 4

D
DAPI - 4', 6'-diamidino-2-phenylindole
DMEM - Dulbecco's Modified Eagle Medium
DNA - Deoxyribonucleic Acid

E
E-CAM - E-Cell Adhesion Molecule
ECM - Extracellular Matrix
EDTA - Ethylenediaminetetraacetic Acid
ESC – Embryonic Stem Cell

F
FACS - Fluorescent Activated Cell Sorting
FBS - Fetal Bovine Serum
FDA - Food and Drug Administration
FI – Fold Increase
FITC - Fluorescein Isothiocyanate
FSC – Forward Scatter

G
G – α-L-Guluronic Acid
GAG - Glicosaminoglycan
GMP – Good Manufacturing Practices
GvHD - Graft-versus-Host Disease

H
HSC – Hematopoietic Stem Cell
HSPC - Hematopoietic Stem/ Progenitor Cells

I
IL-4 – Interleukin-4
IL-6 – Interleukin-6
iPSC – Induced Pluripotent Stem Cell
ISCT - International Society for Cellular Therapy

L
LDH – Lactate Dehydrogenase

M
MFI – Mean Fluorescence Intensity
MHC-I - Major Histocompatibility Complex-I
MHC-II – Major Histocompatibility Complex-II
MI – Myocardial Infarction
MNC - Mononuclear Cell
MSC - Mesenchymal Stem/ Stromal Cells

O
O_2 - Oxygen

P
PBS – Phosphate Buffered Saline
PE – Phycoerythrin
PFA – Paraformaldehyde

R
RNA - Ribonucleic Acid
ROS - Reactive Oxygen Species
RT – Room Temperature

S
SCF - Stem Cell Factor
SD – Standard Deviation
SEM – Standard Error of Mean
SSC – Side Scatter

U
UC - Umbilical Cord
UCB - Umbilical Cord Blood
UCM – Umbilical Cord Matrix

V

VCAM-1 – Vascular Cell Adhesion Molecule 1

VEGF - Vascular Endothelial Growth Factor

X

Xeno-free – xenogeneic free

Chapter I

Introduction

Chapter I - Introduction

In light that a single cell has the ability to develop and create a whole functioning organism, makes us wonder how deep we can manipulate cells in order to surpass biological hurdles, and eventually improve existing medicines/therapies and health.

With the objective to go further away from pure chemical therapies and more into biological approaches (including medical devices, complex biological molecules as well as cell-based therapies), the study of stem cells has never been more demanding (Mason and Manzotti, 2010). Due to their unique abilities of self-renewal or differentiate into multiple lineages, stem cells have given evidences of a versatile tailoring for many applications, where the success lies on how well the scientist can manipulate those cells.

Throughout this thesis, the main focus is a particular type of cells: mesenchymal stem/stromal cells (MSC). This chapter is divided into sections focusing what are MSC, what are the possible applications for such cells, and what are the challenges for producing these in a controlled and safe manner.

I.1. Stem cells

Having huge potential and relevance for clinical applications, stem cells have been growing in interest on both scientific and clinical communities (Kinney et al., 2011; Kondo et al., 2003). The criteria to define these cells are: having the capability to self-renew maintaining a undifferentiated state and also being able to specialize by differentiating into multiple cell types (Frith et al., 2009). More clearly, these cells have the ability to generate daughter cells with identical genetic code to their mother, or generate a cell with a higher level of commitment towards a specific lineage of interest (Lanza et al., 2005).

Although stem cells can give rise to different lineages, this type of cells is not exclusive of embryos/infants. In fact, they can be found in the various stages of life including adulthood, being divided into four classes according to their origin: embryonic stem cells (ESC), fetal stem cells (FSC), neonatal stem cells (including cells from the placenta and umbilical cord) and adult stem cells, as presented in Figure 1 (Bongso and Lee, 2005).

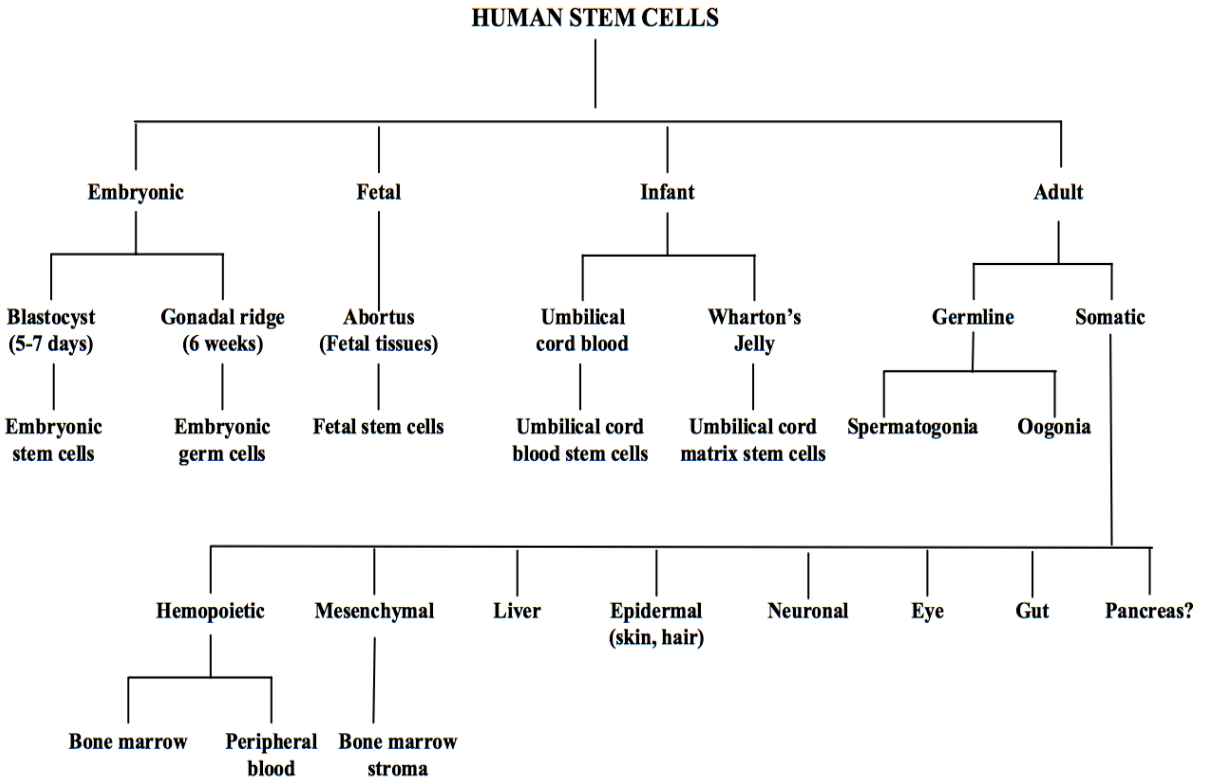

Figure 1 – Classification of stem cells according to their origin. From (Bongso and Lee, 2005).

Stem cells can also be classified according to their differentiation potency: totipotent, pluripotent, multipotent and unipotent. Totipotent stem cells – generated from the fusion of an egg and a sperm cell these cells and few further cell divisions are able to give rise to all embryonic and extra-embryonic cell types, being able to generate a whole organism; pluripotent stem cells – derived from totipotent cells these are able to give rise to all three germ layers (endoderm, mesoderm and ectoderm). The main difference lies in the inability of these cells to give rise to extra-embryonic cells; multipotent stem cells – able to give rise to a subset of cell lineages, generating progenitor populations that can then generate mature cells. This type of cells are the major contributor for tissue homeostasis; and unipotent stem cells (*e.g.* testis stem cells) – able to contribute to only one mature cell type, but retain the self-renew property which set them apart from non-stem cells (Jiang et al., 2002; Mitalipov and Wolf, 2009; Wagers and Weissman, 2004).

ESC alongside with FSC, associated to an earlier stage of development compared to adult cells are pluripotent stem cells, hence holding great promise. However, the procurement of these cells raises huge ethical concerns which present a hurdle in academia (Smith, 2001;

Yu and Thomson, 2011). Moreover, the tumorigenic potential associated to ESC augments the concerns with safety topics (Watt and Driskell, 2010). On the opposite side of the spectrum, Umbilical Cord Blood/Matrix (UCB/UCM) and Adipose Stem Cell (ASC), which are further removed from ethical dilemma as most of the sources are even considered biological waste from other medical procedures (Young and Black, 2004). These, however, are multipotent stem cells, with a more restricted differentiation potential, but may have a more direct route to clinical translation (Atala et al., 2011). UCB/UCM are a neonatal source of stem cells, which are readily available after childbirth. ASC are undifferentiated cells that have a more limited number of lineages to which they can differentiate into, and are found in almost every adult human tissue or organs, promoting homeostasis (Körbling and Estrov, 2003; Mimeault et al., 2007). In order to do this, ASC divide towards the generation of progenitor cells that then specialize into tissue-specific cell types with more specific functions. These cells serve to replace cells that died due to diseases or injuries (Bonnet, 2003; Mimeault and Batra, 2006).

ASC can be found in multiple tissues, and some examples are endothelial progenitor cells, neural stem cells, hematopoietic stem cells (HSC) and mesenchymal stem/stromal cells (MSC). The best characterized source of ASC is from bone marrow (BM), where both HSC and MSC can be found (Caplan and Bruder, 2001). However, MSC can be found in adipose tissue (AT), liver, skin and blood.

Considering the relative ease of isolation, from various tissues, and ease of expansion *in vitro*, MSC interest has grown significantly. Moreover, the properties of these cells may offer new possible therapeutics, making a suitable candidate for clinical use.

I.2. Mesenchymal Stem/Stromal Cells

The first stromal cells isolated by adherence where performed by Friedenstein and colleagues back in 1970's, where they reported the existence of "marrow stromal fibroblasts" from BM (Friedenstein et al., 1970; Owen and Friedenstein, 1988). Later, this concept evolved as the population of adult stem cells found in BM that was not from hematopoietic origin, was named mesenchymal stem/stromal cells (MSC).

This type of stem cells are characterized by being multipotent, deriving from the mesoderm germ layer, and having the ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages (Caplan and Bruder, 2001). Additionally, these cells present low expression levels of the Major Histocampatibility Complex-I (MHC-I) and negligible levels of

MHC-II, rendering these cells low-immunogenic, which enhances their usage for transplantion and tissue repair applications (Chamberlain et al., 2007; Deuse et al., 2011). Furthermore, the new trend with the application of MSC consists in immunomodulation, where instead of relying on these cells to repair the injuries by direct cell replacement, they can secret trophic factors involved in tissue repair and enhance wound healing (Summer and Fine, 2008). By modulation of their local microenvironment, MSC can tailor paracrine activity to promote angiogenesis, prevent apoptosis, modulate immune response, increase tissue regeneration and support the expansion/differentiation of hematopoietic stem/progenitor cells (HSPC) (Prockop et al., 2010).

Another feature of interest that will be explored further along this thesis is the easy of collection and the fewer ethical issues surrounding these cell population. Despite BM has been, due to historical reasons, the primary source of MSC, these cells can be found in different tissues and are now being isolated from several neonatal and adult sources, such as umbilical cord matrix, placenta, amniotic fluid, adipose tissue, dental pulp and synovial fluid (Caplan, 2011; Fukuchi et al., 2004; Gronthos et al., 2002; Jones et al., 2004; Kern et al., 2006; Zhang et al., 2011). Although they all share the same definition, it has been established that the source plays a major role in the characteristics of the MSC, meaning that depending on the source they can diverge in frequency, proliferative capacity and level of commitment to a determined lineage (Klingemann et al., 2008; Musina et al., 2006).

In an effort to standardize the definition of MSC, the International Society for Cellular Therapy (ISCT), established minimal criteria for classifying a stem cell into this category. It does not exist a single marker that establishes a cell as a MSC, but rather a set of criteria that makes it so. Cells should present *in vitro* plastic adherence, should express some cell surface markers (CD73, CD90 and CD105) and not express others (CD14 or CD11b, CD45, CD34, CD79 or CD19, and HLA-DR), and must have the ability to differentiate into osteogenic, adipogenic and chondrogenic lineage by specific *in vitro* stimuli (Dominici et al., 2006).

I.2.1. Cell properties and cell niche

As mentioned above, MSC have the ability to adhere to a polystyrene surface, making their appearance resemble fibroblast cells, which have a small cell body with elongated extremities (Chamberlain et al., 2007). Despite an initial lag phase after isolation or passage, these cells divide rapidly depending on the source, plating density, cell passage and media used.

Phenotypically, MSC do not express a distinctive marker that allows for its identification, but rather a set of markers that has been established to identify this population. Among the positive (CD73, CD90 and CD105): CD73 is an ecto-5'-nucleotidase which recent studies point towards contribution to cell and tissue stress responses; CD90 is a surface protein related to cell-cell and cell-matrix interactions with roles in T-cell activation, wound healing, among others; and CD105 is an endoglin that plays a crucial role in angiogenesis (Colgan et al., 2006; Moraes et al., 2016). On top of the ones established by the ISCT, reports shown that MSC can also express CD44 and Stro-1 (typically for BM source), as well as adhesion molecules CD106, CD166 and CD29. On the other hand, MSC must lack a set of "negative markers" including a series of hematopoietic markers (CD45, CD34 and CD14 or CD11b) and MHC-II surface receptor (HLA-DR). Additionally, other markers were also reported to not be present in MSC, such as endothelial adhesion molecule (CD31), costimulatory markers (CD80, CD86 and CD40), leukocyte function-associated antigen (CD18), nor neuronal cell adhesion molecule (CD56) (Chamberlain et al., 2007). Despite this, consensus is still far from reached in terms of which characteristics define MSC, especially markers, because they can vary from tissue source, isolation method, culture conditions and other (Javazon et al., 2004). The absence of expression of MHC-II and costimulatory molecules by MSC is a fundamental factor to allow these cells to be used in clinical applications. The reason behind this is that they present low/no immunogenicity and so abdicate the need for immunosuppression, allowing their use in a allogeneic setting (Ben-Ami et al., 2011).

It is important to mention the manner these cells are kept *in vivo*, as it has some significant differences to *in vitro* culture. Regarding this point, BM, being a primary source, only comprises 0.001-0.01% BM MSC of total nucleated cells (Pittenger et al., 1999). Not only various types of cells comprise the BM niche, but also other biological and physiological cues take action. Apart from other types of cells, one element that plays a decisive role is the extracellular matrix (ECM), which allows cell-cell interactions, cell-matrix interactions, that affects MSC survival and function. Not only the components of ECM are important, but also the structure it provides, conferring a 3-D organization that assists cell fate. Another main difference lies on the oxygen tension, where in the BM niche is closer to 5% O_2, instead of the 21% O_2 commonly used *in vitro* (Spencer et al., 2014). When cultured under hypoxia, cells not only present different growth patterns but also altered secretory and angiogenic potential, which could be important when using MSC for immunomodulatory applications (Eliasson and Jönsson, 2010; Santos, 2014).

I.3. Immunomodulatory effects and trophic activity

Although not yet fully understood, immunomodulation mechanisms allows MSC to play a role in e T-cell proliferation, dendritic cell maturation, suppression of B-cells, among others (Hong et al., 2012). Also, the immunomodulatory properties of MSC have been used to treat patients with graft-versus-host disease (GvHD), the most common deadly complication caused by allogeneic hematopoietic cell transplants (Baron et al., 2010). These immunomodulatory features are not only due to cell-cell contact but also for paracrine signalling through cytokines and other soluble factors, allowing MSC to sustain a support role for hematopoietic cells (Majumdar et al., 2000; Summer and Fine, 2008).

Both the hypo-immunogenicity for allogenic applications and anti-inflamatory properties are appealing factors for the use of MSC for treatment of injuries and immune diseases, but also the capacity of these cells to target damaged tissues has shown more clinical interest (English et al., 2010). Among others, vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), hepatocyte growth factor (HGF), prostaglandin-E2 (PGE2), transforming growth factor-β (TGF-β), and tumour necrosis factor (TNF)-stimulated gene 6 (TSG-6), are some of the factors that play roles in tissue regeneration, anti-scarring, anti-apoptotic signalling, proliferation and differentiation, inflammatory reaction and angiogenesis (Ben-Ami et al., 2011; Caplan and Correa, 2011).

I.4. Sources of Mesenchymal Stem/stromal Cells

As previously mentioned, MSC can be found in many tissues, in particular in BM, UCM and AT (Figure 2), three cell sources used in this work, which will be discussed further herein. Cells from all sources, despite being all defined as MSC, differ in, for example, availability, proliferation potential, as well as ECM and cytokine production. More technical restraints also separate the different sources, regarding easiness to obtain samples, contamination risk or MSC yield (Klingemann et al., 2008).

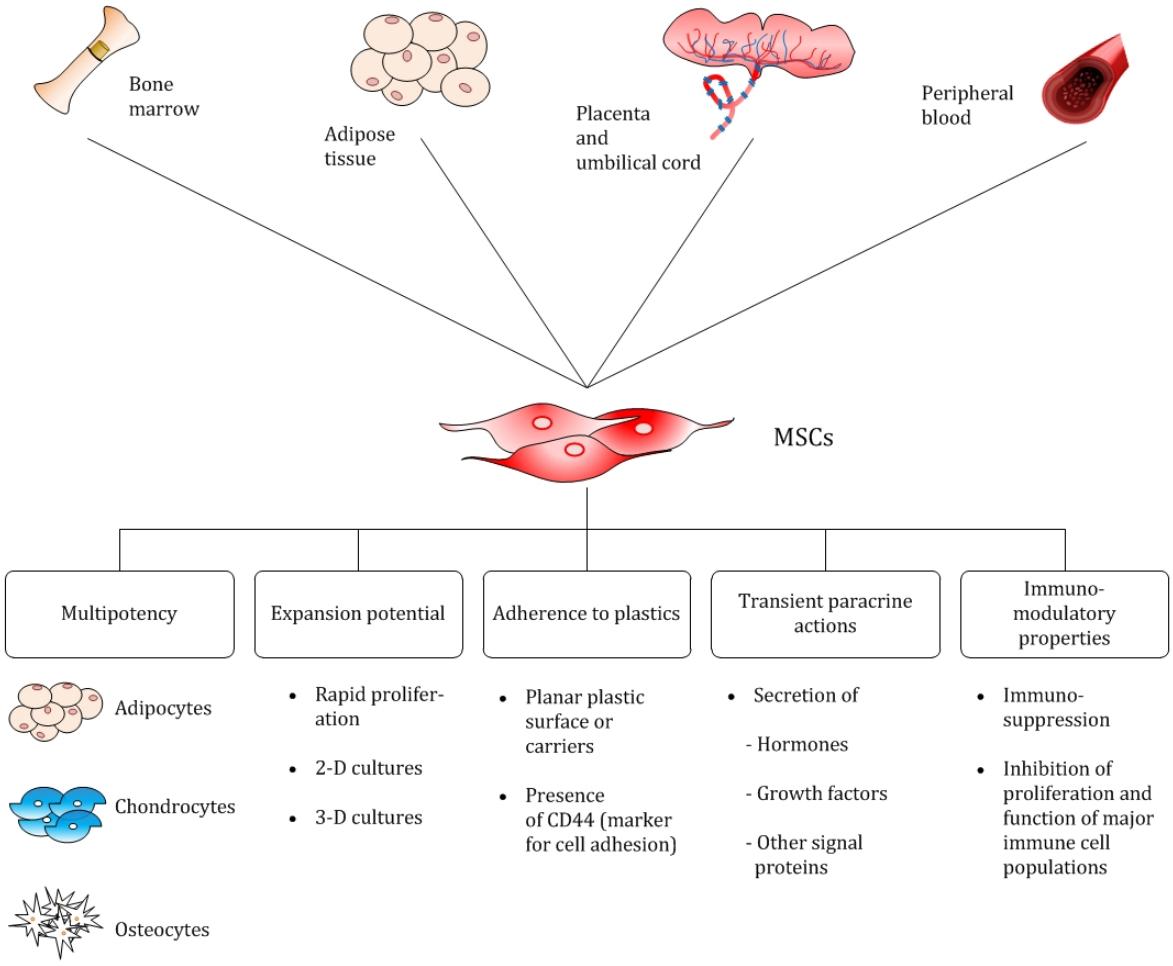

Figure 2 – Schematic representation of mesenchymal stem/stromal cells sources and their characteristics. MSC clinical interest is due not only with their ability to differentiate in a variety of tissues but also for they immunomodulatory properties and paracrine secretion of angiogenic factors, hormones and cytokines. From (Jossen et al., 2014)

It is uncertain if MSC from different sources have distinct functions as ASC that could be exploited into different medical applications. Moreover, it was shown that they report differences in genomic levels that could influence their role (Klingemann et al., 2008). In

common with all sources is the final step of isolation, where cells are typically plated on plastic surface and MSC will adhere while most contaminants will remain in suspension. One of the major disadvantage of this step is the presence of adherent cells of hematopoietic origin called monocytes, usually observed from BM source. As alternative for plastic adherence, one could use Magnetic-activated cell sorting (MACS) or Fluorescence-activated cell sorting (FACS) for a more controlled cell sorting. These techniques use specific surface markers found in MSC in order to sort them from contaminants, which are highly reliable, however the elevated cost of these methods presents another obstacle.

I.4.1. Bone Marrow

The first know source of MSC and the most extensively studied source is BM. This consists in a hematopoietic tissue that is situated inside the trabecular bone, being responsible for the maintenance of the hematopoiesis – production of all hematopoietic cells. This process is supported by the BM stroma, which contains fibroblasts, osteocytes, osteoclasts, adipocytes, macrophages, vascular endothelial cells and ECM (Moore and Lemischka, 2006). BM niche contains both HSC and endothelial progenitor cells (EPC), apart from the MSC which is a small percentage of total cell population (Pittenger et al., 1999). Nonetheless, MSC have the function of providing support for hematopoiesis by providing ECM components, cytokines and other growth factors (Atala et al., 2011). The oxygen tension inside BM niche varies from 0.1% in endosteal region and 6% near blood vessels, making it a complex gradient that plays a role influencing BM MSC (Parmar et al., 2007).

The harvesting method for a BM cells is an invasive and painful technique, that involves anaesthesia. Using a syringe to perform a BM biopsy, the marrow is aspirated from the iliac crest of the pelvis. Then, a density gradient is performed (e.g. using Ficoll), that is used to separate the cell components of the blood. The mononucleated cells are then plated and cultured on plastic culture flasks, in order to further remove other contaminants that do not possess the ability to adhere to plastic (Barry and Murphy, 2004; Kern et al., 2006). After this process the yields obtained from isolation from BM aspiration are very low, highlighting the need for expansion before usage in any clinical setting (Gronthos et al., 2003). BM MSC have the limitation of decreasing significantly the frequency, proliferative capability and differentiation potential as the donor age increases (Ra et al., 2011).

A feature of interest of this source is the higher production, when compared with other sources, demonstrated by Klingemann and colleagues of VEGF (and SDF-1), factor essential

for EPC differentiation, which indicates the suitability of BM MSC to support angiogenesis. SDF-1 is also related to homing process of MSC as it binds to CRCX4 in cells (Klingemann et al., 2008).

Considering the drawbacks of BM collection and the particular features that these MSC present, alternative sources could be advantageous in both isolation easiness and yield, as well as other applications that could benefit from a particular feature of that source.

I.4.2. Umbilical Cord Matrix

The blood that remains in the placenta and umbilical cord (UC) is a prime source for isolation of HSC, but MSC can be collected from the umbilical cord tissue or matrix. Establishing a connection between the embryo/fetus and the placenta, the UC is composed by two arteries and one vein buried within Wharton's jelly (Fan et al., 2011). Wharton's jelly is a mucous connective tissue composed by fibroblast and occasionally mast cells surrounded by an amorphous ground substrate rich in collagen (type I, III, IV, VI and VII) and glycosaminoglycans (GAGs) (Qiao et al., 2008). Considering the highly coiled structure of UC, this tissue has the purpose of averting compression, torsion and bending of the vessels (Can and Karahuseyinoglu, 2007).

The availability of the UC does not raise major ethical concerns, and is even usually considered medical waste, readily disposed by hospitals (Anzalone et al., 2010). Deprived of any invasive procedure to collect this sample, after removal of the vein and arteries the Wharton's jelly of UCM can be broken down with collagenase type II in order to release the entrapped MSC (Simões et al., 2013). Other studies have shown the possibility to place minced pieces of Wharton's jelly T-flasks and MSC will migrate onto the plastic surface, bypassing the enzymatic digestion (De Bruyn et al., 2010). Despite the ease of sample procurement, the major hurdle linked to this source lies on the low yield of cells at the end of the process, which stray far from clinical relevant numbers (Atala et al., 2011; Zeddou et al., 2010).

Despite being able to undergo differentiation into the three lineages, UCM MSC differentiate into chondrocytes slower than BM MSC. However, these cells produce higher levels of hematopoietic growth factors granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor, angiogenic factor interleukin-8 and activator of immune responses interleukine-1 (Klingemann et al., 2008). Moreover, UCM MSC present lower risk of both of genomic alterations resultant of aging or diseases, as well as lower risk of viral contaminants. A major feature from this source is that, due to their more primitive stage

when compared to ASC, they present shorter doubling times and great number of passages before senescence is observed.

I.4.3. Adipose Tissue

Adipose tissue is a loose connective tissue situated beneath the skin, around the organs and blood vessels, in bone marrow and breast tissue. It is composed mainly by fibroblasts, macrophages, endothelial cells, adipocytes and, more recently identified, MSC. Upon collection of sample, the stromal vascular fraction is separated and digested with collagenase type I, rendering a population of cells that can further separated by using the MSC plastic adherence property (Kern et al., 2006).

AT represent the second most common source of MSC being used clinically, closely to BM source (Kern et al., 2006). Samples of fat tissue are obtained through other procedures performed such as liposuction (Schäffler and Büchler, 2007). Although it is an invasive process (still less invasive than BM), it is a by-product from other medical interventions considered medical waste (Atala et al., 2010). Nevertheless, unlike the previous mentioned sources, AT MSC can be retrieved in high numbers, as AT is not restricted to a small place (BM) or event (childbirth). Moreover, AT contains larger quantities of MSC than BM – 1 x 10^5 MSC per gram of fat (Ra et al., 2011; Ringdén et al., 2006).

In addition, AT MSC have high proliferative potential and higher culture period before reaching senescence state, when compared to BM (Kern et al., 2006). These cells do not express CD106 and Stro-1, which are expressed in BM MSC, and express CD49d (Klingemann et al., 2008).

I.5. Therapeutic features and clinical applications of MSC

In regenerative medicine, when using cellular therapy or performing organ transplants one of the main problems faced is the triggering of an immune response from the patient that leads to the rejection to cells/organs (Jones and McTaggart, 2008). The two options available to overcome this obstacle is either use autologous cells or immunosuppress the patient, both with risks. Autologous cells present problems regarding the collection of these cells from the patient, which could be troublesome considering some cases where the patients are in severe

conditions, with comorbidities, patients could not have enough time to obtain autologous MSC and culture them, or even patients being immunocompromised and allogeneic MSC being the only option (Dazzi and Krampera, 2017).

Moreover, cells from the patient might not be suitable in all cases due to a genetic disease or the need for expanding this cells into a clinical relevant number (Atoui and Chiu, 2012). There is a need for an allogeneic off-the-self product that could overcome these problems. MSC have the advantage of being immunoevasive , which might allow for a patient to receive cells from a non-matched donor without triggering an immune response (Ryan et al., 2005). Thus, allogeneic MSC from healthy donors can be used to treat a set of diseases with an off-the-self product, or even as a support product to be used combined with other treatment in order to evade immune response (Klingemann et al., 2008).

Apart from the ability of MSC to differentiate into osteogenic, chondrogenic and adipogenic lineages, the trophic potential of MSC to tackle different diseases have been discussed recently, opening new applications for this type of cells in regenerative medicine (Burdon et al., 2010; Prockop et al., 2010). MSC have demonstrated their trophic activity in angiogenesis, wound repair and immunomodulation and their response to external stimuli through signalling pathways. Several clinical trials are undergoing using MSC therapies in order to tackle diseases such as GvHD, myocardial infarction and amyotrophic lateral sclerosis.

I.5.1. Wound repair

Due to their immunomodulatory and tissue regeneration properties, MSC contribute to repair damage tissue and are already being applied in various clinical trials (Hocking and Gibran, 2010). However, because of the low survival of MSC at injured sites, these cells resort to paracrine signaling that regulates local cellular action and is considered the principal mechanism used by MSC to respond to injuries. Throughout the wound healing process, MSC produce trophic factors, including anti-inflammatory cytokines, promote angiogenesis and recruit cells to aid in the process (Hocking and Gibran, 2010). On top of that, MSC are also capable to produce components of the ECM, essential to restore the integrity of the damaged tissues. Recruitment of fibroblasts, immune cells and endothelial cells to the wounded site is done via MSC by the secretion of VEFG and HGF (Chen et al., 2016).

On the other hand, MSC secreted factors can be used as a conditioned medium (CM) instead of the cells itself. Due to the low engraftment of cells in the injured site and the hurdles of implementing a cellular therapy, the usage of CM have been exploited in the last years

(Eggenhofer et al., 2012; Santos et al., 2015). The objective is to mimic the *in vivo* secretome of MSC, but not only is a very complex mixture of factors, but also the highly concentrated factors already present in culture medium used can hinder the MSC effects. A major hurdle is the fact that the mechanisms behind the regenerative features of MSC are not fully understood, meaning that trying to replicate a specific cocktail of factors built by a complex network of cells for a specific time-frame is very challenging.

I.5.2. Tissue engineering and immune diseases

MSC are already encoded to maintain the homeostasis of the tissues such as bone marrow, gastrointestinal track and skin. However, some tissues, like cardiac muscle for example, have a very limited ability for tissue repair. Hence, tissue engineering is a direct approach to repair of injured bone, joint, muscle or cartilage (Javazon et al., 2004). As these tissues are limited for regeneration and lack structure for the repair, scaffolds are being used in order to aid in tissue engineering. The scaffold structure provides the microenvironment and cues for the MSC to differentiate into the needed tissue and allow the construct (cells plus scaffold) to be integrated in the damaged area.

The immunomodulatory property of MSC implies the secretion of several factors that act in a paracrine manner regulating the immune response of macrophages, NK cells, dendritic cells, B-lymphocytes and T-lymphocytes (Prockop and Oh, 2012). This characteristic allows a better response for inflammatory injuries, autoimmune diseases and helps with co-transplantations (Klopp et al., 2011; Maxson et al., 2012; Uccelli et al., 2008).

MSC-based therapies proved to reduce in-site inflammation, apoptosis and fibrosis in different disease models in spite of the low engraftment of MSC in the injure tissue. The regeneration ability due to trophic factors opens another application of MSC for the treatment of autoimmune diseases (van Poll et al., 2008). These factors, instead of affecting a target area for a short time period, act on the entire system in a systemic way. Examples of such diseases are the previously mentioned GvHD or multiple sclerosis. There are no cures available for these diseases and the current treatments comprises immunosuppressors, increasing the chance of other complications (Atala et al., 2011). In fact, the ability of MSC to home to sites of inflammation and modulate immune responses, while being evasive to the immune system, gives an important advantage when compared to other current therapies (Wang and Zhao, 2009). Although using systemic administrations of MSC to manage immune diseases is less

invasive, the challenge is how to assure the infused cells reach the target tissue. Adjusting the dosage and the dosing regimen is a critical step to deal with cell loss, and current clinical trials vary from $1x10^6$ to $5x10^6$ cells per kg of patient body weight (Santos et al., 2011). Less than 1% of infused MSC is reported to reach the target tissue mainly due to entrapment in capillaries within liver and lungs. This event occurs because of the large size of cultured MSC and various adhesion receptors on the surface of these cells that binds to lung tissue (Ankrum and Karp, 2010). Reports shown that in fact MSC are not able to home to bone marrow in an efficient manner (Schrepfer et al., 2007). On top of the entrapment, cells that reach the damaged tissue are subjected to a harsh environment and their survivability is another challenge. Because of the extreme inflammatory response and the lack of ECM to the oxidative stress, MSC tend to die in injured sites, diminishing their time to respond and aid in tissue regeneration. *In vivo*, among other factors, the accessibility to oxygen is hindered in damaged ischemic tissues leading to MSC failing to reproduce the *in vitro* results (Song et al., 2010).

One of the strategies applied to overcome the low homing and engraftment of MSC to target tissues is the increasing of the number of administrated cells. Although the direct relation between number of injected cells and number of cells found in the target tissue exists, the already high number of cells needed poses a major bottleneck (De Becker and Van Riet, 2016). Hence, a subtler approach using the pre-conditioning of MSC to overexpress "homing receptors" (such as adhesion molecules) in order to increase the probability of infused cells to reach the therapeutic site (De Becker and Van Riet, 2016; Huang et al., 2010; Nitzsche et al., 2017).

I.6. Culture Systems for Mesenchymal Stem/Stromal Cell expansion

As previously discussed, MSC are important candidates for regenerative medicine and cellular therapy applications. However, the number of cells required for a clinical application can go up to $5x10^6$ cells per kg of patient body weight (Santos et al., 2011), a number that far exceeds the number that can be isolated from any of the sources available, meaning that there is a need for *ex vivo* expansion protocols that can reach the required number of cells in a reproducible manner. MSC can be expanded up to 40 generations whilst maintaining they differentiation potential, but the mitosis rate diminishes considerable and cells enter a senescence state (Deans and Moseley, 2017). Therefore, there is a need for rapid efficient expansion protocols that can expand MSC while maintaining the inherent attractive properties.

The understanding of MSC biology and its role in the various process that are involved, allows a more controlled and reproducible method for expansion.

Not only the objective is to allow the expansion of MSC, but also the removal of all contaminants that may confer a level of uncertainty to the final product. This task if striving due to the lack of specific MSC markers. Moreover, MSC need to retain/enhance the ability survive after infusion and be able to home into the target tissue in order to aid in the regeneration. For that, microenvironment and structural cues need to be taken in consideration *ex vivo*.

Different strategies have been used for expansion and fine tune of MSC properties to serve the intended purpose. However, the traditional methodology used for expansion is a two-dimensional (2D) approach, which contrasts with the three-dimensional (3D) organization found in the BM niche, UC or even fat tissue. MSC expansion at a clinical-scale is traditionally carried out under static conditions but, as an alternative, 3D cultivation in bioreactors are being studied and used to increase cell expansion rates and are easier to control in order to fine tune cells.

MSC are adherent cells, which means that it is required a substrate where these cells can attach on. When considering 2D culture, treated polystyrene is the gold standard, but for 3D cultures the options vary considerably (Yuan et al., 2014). Cell adherence is promoted not only by the surface but also by the medium used for culture, which can have a considerable impact for a culture success. Culture surface can be coated with poly-D-lysine, gelatin, collagen, laminin and fibronectin, which not all enhance cell adhesion and then proliferation but rather play a role in differentiation cues or changes in cell morphology (Qian and Saltzman, 2004; Sotiropoulou et al., 2006).

Cell density is a critical parameter in order to assure an equilibrium between allowing maximum cell expansion and not compromising cell-to-cell interaction. However, reducing seeding densities leads to an increase in the proliferation rate and population doublings (Ankrum and Karp, 2010). On top of that soluble factors, mechanical forces, oxygen tension and culture handling, play a role in cell culture (dos Santos et al., 2010). Specifically, oxygen tension is a key and sometimes overlook factor for MSC proliferation, as normally MSC are cultured under 21% O_2 (referred to as normoxia), but the BM niche is known to have approximately 2% O_2. Grayson and colleagues showed that MSC under hypoxic conditions (2% O_2) maintained their ability to proliferate and expressed higher levels of stem cell genes than when compared with normoxia (Grayson et al., 2006). Moreover, oxygen tension has been shown to accelerate cell proliferation kinetics and enhance efficiency of MSC metabolism (dos Santos et al., 2010).

I.6.1. Two-dimensional culture

The gold standard for MSC cultivation is a two-dimensional static culture, for instances a T-flask, made of polystyrene, represented in Figure 3. These devices are simple, easy to handle and can be stacked in incubators. MSC are isolated/thawed and plated on T-flasks in a monolayer culture, while the incubators allow the control of temperature and a humidified atmosphere with 5% CO_2. The medium must be changed at a regular basis of three to four days and checked under microscope for confluence. At around 80% confluence, cells are detached using an enzymatic reagent and are then replated on a higher number of new T-flasks for further expansion (Santos et al., 2011). MSC can be passaged *in vitro* between 8-15 passages depending on the source, media and cell density used, up until 40 population doublings before presenting senescence (Wagner and Ho, 2007).

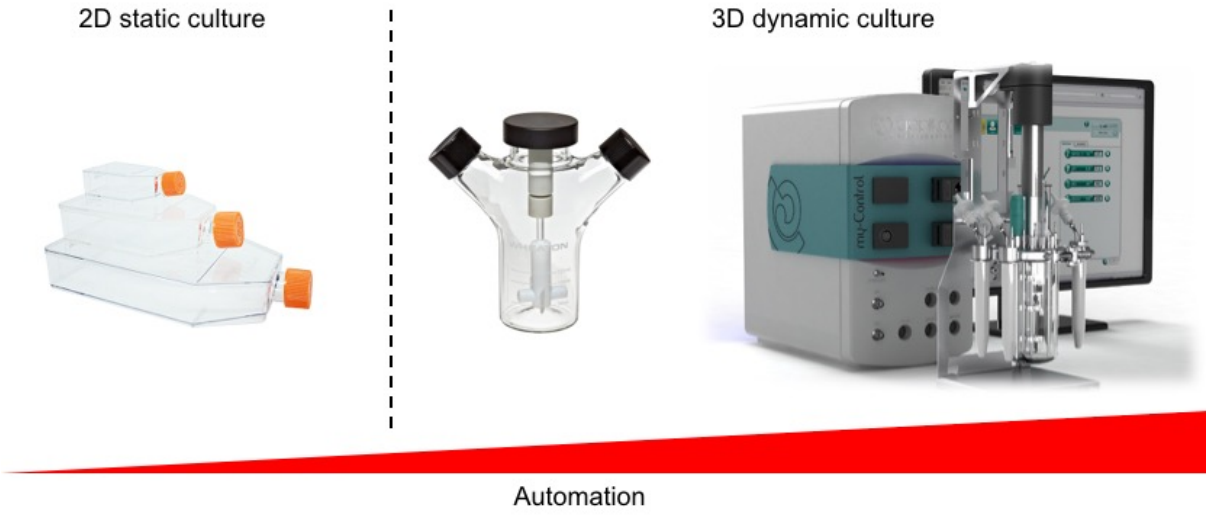

Figure 3 – Representation of T-flasks (left), spinner flaks (centre) and Applikon mini-bioreactor (right). As culture system goes from left to right, the labour decreases due to system automation. Dynamic 3D cultures require surface support for mesenchymal stem/stromal cells adherence such as spheroids or microcarriers. From (Applikon Biotechnology, 2017; Vaccixell, 2017; Wheaton, 2017)

Despite of already being optimized, 2D cultures present some limitations in terms of cell yields, lack of information and control regarding culture parameters, continuous and laborious handling and long cultivation periods. The latter drawback, in combination with the multiple passaging required, increases the risk of error, contamination of culture or undesired genetic modifications (Santos et al., 2011). The handling or these cells brings out another

difficulty concerning the culture parameters. Although incubators allow control over some parameters, the handling of the cells in between passages is performed inside a laminar flow chamber, which although sterile, offers no control over temperature, humidification nor oxygen tension.

In order to try and tackle the demands of the clinical settings while using 2D culture, multi-layered cell factories have been using to culture MSC (Connick et al., 2012). These simply increase the size and number of polystyrene stacks, offering a larger surface area. However, these retain the inherent limitations is a static 2D culture, including the impossibility to monitor and control parameters such as nutrients, pH and dissolved O_2 (Randers-Eichhorn et al., 1996).

Although MSC retain their plasticity for several passages in a 2D culture, it has been reported that before reaching the clinical relevant numbers, the cells have a significant loss in proliferation ability, ability to self-renew, ability to form colonies and even their differentiation potential (Baraniak and McDevitt, 2012). On top of this, the usage of 2D surfaces forces MSC into a flat conformation that does not mimic the cell-to-cell and cell-matrix interactions that can be observed *in vivo* (Saleh and Genever, 2011). Moreover, the properties of polystyrene surface do not correlate in any way with the ECM found in BM niche, "pervading" cell fate and being described to promote the spreading of cells rich in actin-myosin stress fibers (Dawson et al., 2008; Discher et al., 2009).

I.6.2. Three-dimensional culture under dynamic conditions

With the objective of overcoming the previously mentioned limitations, 3D culture techniques, namely using microcarriers, or alternatively cell cultivation as aggregates (see section I.7), in suspension, have been applied in several studies. Also, the need to attained a significant larger number of cells calls for a protocol that could be scaled-up in a robust, efficient and cost-effective manner, and compliant with Good Manufacturing Practice (GMP) standards (Eibes et al., 2010). A fully controlled bioreactor is a system that would be able to meet the demands of cell production, while minimizing the risks associated with contamination (Yeatts and Fisher, 2011). From spinner flasks, stirred bioreactors, rotating wall vessels, concentric cylinder bioreactors, packed bed bioreactors and perfusion bioreactors, all platforms have been tested for MSC culture presenting its own advantages and drawbacks (Frith et al., 2009; Weber et al., 2010; Zhao and Ma, 2005).

Spinner flasks are a not fully controlled culture system that allows some automation. These consist in bottles with a magnetic stirrer to improve homogeneity of suspension cultures. In this dynamic culture, the medium is continuously mixed gaining a higher productivity and efficiency by eliminating the concentration gradients of pH, dissolved oxygen, growth factors and metabolites (Eibes et al., 2010; Santos et al., 2011). In spite of the cells benefit from low shear stress, improved supply of nutrients and outflow of wastes, hydrodynamic shear stress has been proven to influence both cell function and cell differentiation (Burdick and Vunjak-Novakovic, 2008; Sart et al., 2010). This culture system, either combined with cell culture on microcarriers or aggregates, still lacks control over most parameters and although reduces handling, the work performed is still done in a flow chamber, undermining the culture conditions established. Still, this type of platform is usually a stepping stone in a scaling-up scheme, targeting a stirred-tank bioreactor.

Nevertheless, these platforms are showing evidences of differences in the phenotype and biological responses of cells when compared with 2D culture (Frith et al., 2009). Although not yet completely understood, the differences noted in morphology, growth rates and even secreted molecules, may come from a combination of factors of cell-to-cell, cell-matrix, shear stress, nutrient gradients, 3D structure and type adhesion surface (Saleh and Genever, 2011).

This type of culture is not only more appealing for the capability to attain more cells but also for the more closely mimicking with the *in vivo* conditions, which is relevant for tissue engineering. Even so, for each application or purpose the design of the bioreactor is a key feature to produce the desired cells.

I.6.2.1. Bioreactors

Fully controlled bioreactors allow tighter control of culture conditions such as temperature, pH, oxygen tension, feeding regime and waste disposal (Yeatts et al., 2013). These platforms can vary its operation regimen from batch, fed-batch or continuous, in order to maximize cell production according to the objectives set (Wang et al., 2009). By selecting the appropriate regimen for the expansion of MSC, it can be prevented the over dilution of growth factors while preventing the accumulation of metabolic by-products, such as lactate which impair cell growth (Schop et al., 2009). Furthermore, stirred bioreactors can feature online control and monitoring of several culture parameters such as pH, oxygenation, leading to lower human involvement, improving reproducibility and cell products standardization.

The most common and already established designs of bioreactors that significantly improve cell culture are the stirred tank (typically employing the use of microcarriers or 3D spheroids for supporting cells in expansion), rotating-wall vessels, perfusion bioreactors and hollow-fiber reactors (Haycock, 2011). Alternatively, a rotating-bed bioreactor has come into play, whereas instead of the of the construct being continuous under circulation from the medium, the construct itself rotates in a static housing (Zhao et al., 2005). Relatively low shear stress conditions are generated why using rotating vessels and stirred bioreactors, as the speed of rotation/agitation can be adjusted. Stirred bioreactors have been used to expand and differentiate different cell types. On the other hand, in the perfusion bioreactor, a convective flow passes through the construct (e.g. scaffold, microcarriers) that makes nutrients readily available and more rapidly outflows wastes (Visconti et al., 2006). Even without a stirrer device, the mechanical forces created by this flow can influence cell behavior.

One possible alternative configuration, that avoids this drawback, is a hollow-fiber reactor, where the convective flow is maintained but the shear stress is reduced (Zhao et al., 2009). Cells are typically seeded around the fibers, which have porous walls, while medium perfuse through the fibers. Hollow-fiber type reactors also present the advantage of not having to use an external support for MSC to adhere while in expansion, greatly reducing the downstream process of recovery of cells (Rojewski et al., 2013).

I.7. Three-dimensional culture supports

MSC are adherent cells, and so when cultured in suspension cultures, as most 3D cultures, they need a physical support where they can be immobilized in order to proliferate. The most common approach is the usage of microcarriers, a physical support matrix that remains in suspension when under sufficient agitation (Levine et al., 1977). The increased surface area-to-volume when compared to 2D culture systems, allows a more feasible scalability of such platforms (Carmelo et al., 2015; Soure et al., 2016). On the other hand, there are the 3D aggregates of MSC referred as spheroids. These 3D constructs were developed with the intent to mimic more closely the MSC niche *in vivo*, compelling cell-to-cell interaction. In the following sections, the advantages and drawbacks of each approach will be addressed.

I.7.1. Spheroids

MSC have the ability to form a sphere-like aggregate without the need for support. The cell adhesion molecules present in the surface allows cells in suspension to self-aggregate into a spheroid structure, as represented in Figure 4. Aggregation of MSC was used as a technique to enhance chondrogenic differentiation, high-throughput screening or to avoid the usage of an external immobilizing element (e.g. microcarrier), that further down the process had to be removed prior to usage of the cells in a clinic setting (Genever, 2010; Rowley et al., 2012; Steinert et al., 2008).

Culturing MSC as 3D spheroids increases cell-to-cell and cell-ECM interactions and have shown to improve cell survival *in* vivo (Baraniak and McDevitt, 2012). Moreover, spheroid cultivated cells have proven able to maintain and even enhance their stem cell characteristics, increasing the expression of anti-inflammatory factors, and boosting the differentiation potential (Baraniak and McDevitt, 2012; Bhang et al., 2012). With the increased expression of Tumour Necrosis Factor α (TNF-α) stimulated gene/protein 6 (TSG-6) and stanniocalcin-1 (STC-1), spheroid cultured MSC could improve the modulation of immune responses and suppress inflammation. Interleukin-24, TNFα-related apoptosis inducing ligand and CD82, tumour suppressor proteins were also reported to be highly express in MSC spheroids, granting a wider range of clinical applications (Bartosh et al., 2010). In the same study, authors report a smaller size of MSC dissociated from spheroids compared to cells retrieved 2D cultures, which could possibly ease the diffusion of cells throughout the tissues upon administration (Bartosh et al., 2010). Additionally, C-X-C Chemokine Receptor type 4 (CXCR4) expression was up-regulated in 3D culture spheroids, which is a surface marker important for cell adhesion and may strongly enhance the homing ability of MSC (Potapova et al., 2008). Even after separated and cultured in 2D, cells previously cultivated in spheroids still retain some level of enhanced secretion of particular factors (Cheng et al., 2013). Another important feature that affects spheroids is the oxygen tension. Spheroids cultured under hypoxic conditions (1% O_2) produce higher amounts of ECM and other factors such as VEGF, hFGF, IL-11 and BMP-2 (Potapova et al., 2007; Shearier et al., 2016). Particularly, spheroid dimension can hinder the oxygen diffusion, as well as nutrient diffusion, to the inner cells of the spheroid. This gradient is more prominent as the size of spheroid increases, with the consequence of leading to an apoptotic/necrotic centre and/or creating a level of heterogeneity (Saleh and Genever, 2011). Spheroids with a diameter higher than 150 μm start to present diffusional problems, rapidly reducing viability (Genever, 2010).

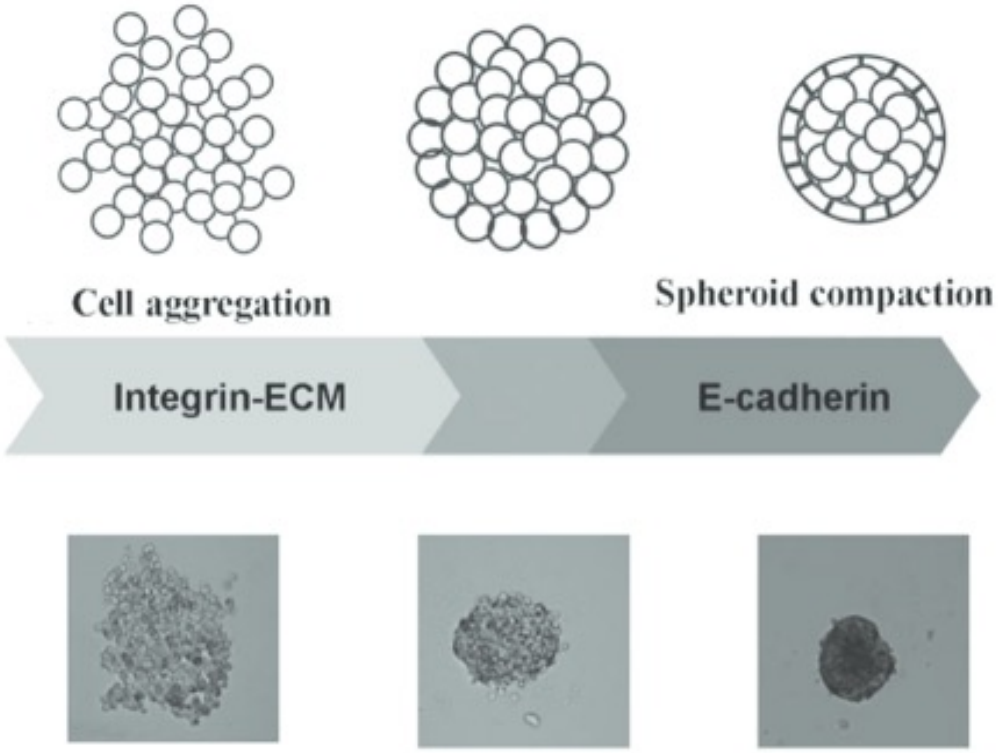

Figure 4 – Mesenchymal stem/stromal cells have the ability to self-aggregate when culture on a non-adherent surface. By increasing cell-to-cell and cell-ECM interactions, the compaction of the spheroid increases, improving the overall survivability in culture. From (Zhou, 2016)

As previously described, MSC have the ability to self-aggregate and from spheroids, however, this method, per se, is unable to control spheroid size effectively. Although the hanging drop, where you force cells to aggregate is the traditional method used for spheroid formation, it still lacks the robustness needed for clinical settings. A more effective strategy involving photolithography to create small but equal microwells was performed (Lin and Chang, 2008). Adjusting the microwell size and the cell density, the number of cell per spheroid can be measured, facilitating the spheroid size control.

However, spheroid cultured MSC have failed in comparison to 2D cultured in their supportive capability of hematopoietic expansion (Schmal et al., 2015). Moreover, spheroid culture have shown some limitations in terms of cell proliferation, not being used particularly for cell expansion (Santos et al., 2015). Hence there is the need to further study the behaviour of MSC while in spheroids in order to improve the culture methodologies. Another setback in terms of spheroid culture is that the size could be a problem for cell infusion in patients, especially if intravenous delivery is envisioned, and thus dissociation of spheroids must be performed (Bartosh et al., 2010). Dependent of the application desired (tissue engineering,

cellular therapy), the type of culture and configuration used for MSC cultivation must be tailored, as there does not exist a unique system to fit all needs.

I.7.2. Microcarriers

With the intent to offer dynamic cultures a large surface to support adherent cell growth, microcarriers were developed by van Wezel. A group of small spherical particles that could be maintained in suspension through agitation could allow anchorage-dependent cells to expand on its surface (Van Wezel, 1967). This allows to create a surface area available for cell growth while maintaining a 3D structure more closely related to the *in vivo* conditions. The usage of microcarriers has increased due the advantages of being easily scalable by increasing microcarrier concentration, high area-to-volume ratio and 3D structure (Chen et al., 2013). This system can be integrated in a fully controlled bioreactor and greatly reduce the handling labour and hence the risks of contamination, as seen in Figure 5.

There are various commercially available microcarriers, and they vary not only in size but also in material, porosity and coating. The microcarriers can be solid, with the cells attached only to the outer surface, or macroporous, with the cells attached to the outer surface and along the walls of the pores (Bleckwenn and Shiloach, 2004). The functional attached coatings are critical for initial cell adhesion and for supporting expansion. From ECM proteins (laminin, fibronectin and collagen), gelatin and positively charged amines are some of the functional groups used to coat the microcarriers to enhance cell expansion, where some increase cell adhesion and others more effectively promote cell expansion. The positive charge conferred to the microcarriers facilitates the attraction of cells through electrostatic forces and improves cellular adhesion. Furthermore, the porosity of microcarriers, as well as the shape, can be made in order to protect cells from shear stress (Chen et al., 2013). Instead of growing on the surface of the bead, cells can grow inside the pores of porous microcarriers (Doyle and Griffiths, 1998).

In order to be able to be maintained in suspension, most spherical microcarriers have diameters between 100-230 μm and densities around 1, but as cell expansion occurs the microcarriers become heavier, and hence higher agitation speed is needed to maintain the homogeneity of the culture. However, shear stress caused by agitation can detach cells from the microcarriers (Chen et al., 2016). Some microcarriers matrices and/or coatings are from animal origin, which inserts the drawback of not being xenogeneic-free (xeno-free). However, several options exist without the usage of animal-derive coatings (Bieback and Kluter, 2007; Tan et al., 2015).

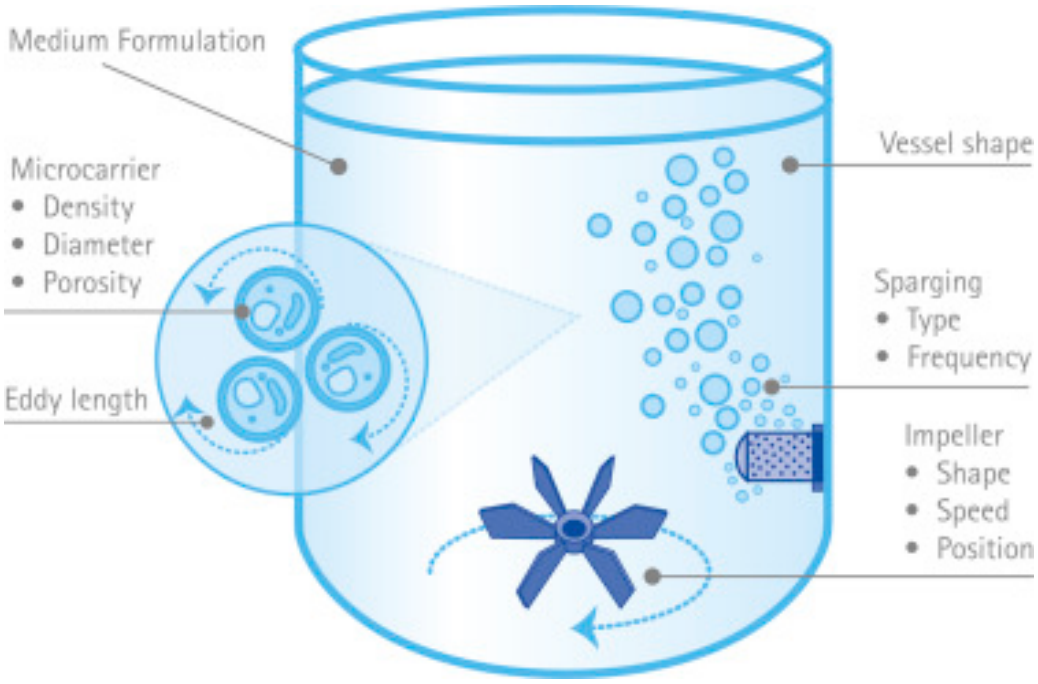

Figure 5 – Schematic representation of microcarrier usage in a stirred-tank bioreactor. Integration of this support technology with a fully automated 3D culture system renders this platform easy of scalability, making it optimal for reaching clinical relevant cell numbers. From (Schnitzler et al., 2016).

The major challenge when using microcarriers as the support for MSC growth lies in the first step of cellular adhesion upon inoculation. Not only the microcarrier type, coating and concentration affect this parameter, but also the cell density, the agitation, pH and the media are major players. However, strategies involving intermittent/lower agitation or coating with adhesive substrates have been used with success for some sources of MSC (Carmelo et al., 2015; Soure et al., 2016). On top of that, having an external particle that must be removed before infusing cells into patients, adds a step of cell recovery in the cell manufacturing process.

I.8. Culture media

Selecting the medium formulation for isolation and expansion of MSC is a crucial step, as medium can influence cell fate. The most common formulation used are composed by a basal medium, which contains nutrients such as glucose and glutamine that cells require to cell processes, and a supplement rich in growth factors and adhesion proteins, such as serum (Sotiropoulou et al., 2006). There are many basal mediums such as Dulbecoo's Modified

Eagle's Medium (DMEM) or Minimum Essential Medium Eagle alpha (αMEM), which choice varies according with the source, supplement added and objective of the culture (Hagmann et al., 2013). There are many variations of these basal media, which can contain more or less nutrients, for example DMEM can have high glucose concentration (4.5 g/l), for a greater availability of carbon source for cell consumption, or low glucose (1 g/l) to limit the proliferation of cell contaminants. For instance, it has been observed that medium with the addition of Glutamax® instead of L-glutamine showed improved results, as the latter is chemically unstable and tends to be rapidly decomposed into ammonia impairing cell growth (Christie and Butler, 1994). Antibiotics are also added to medium in order to prevent contamination by bacteria/fungi while handling of the cells.

The main metabolic pathway of proliferating cells is aerobic glycolysis, which leaves lactate and ammonia as by-products of the metabolism (dos Santos et al., 2010). The lactate accumulation, for example, leads to a pH decrease that could potentially inhibit cell growth. In order to be able to maintain the range of pH within the culture, the basal medium either contains a buffer system or one, such as HEPES, could be added.

Considering the source of growth factors and adhesive factors, the gold standard is basal medium supplementation with 5-10% fetal bovine serum (FBS), as this is a rich and complex source of growth factors, hormones, binding and transport and other nutrients needed for cellular growth (Kern et al., 2006; Oikonomopoulos et al., 2015). However, in regard to clinical translation, FBS presents the disadvantages of being an ill-defined, with limited information on growth factors and cytokine content, having high lot-to-lot variability, being prone to promote xenogeneic immune reactions, and having a high risk of contamination with virus and prions, and thus scientific and clinical communities are trying to refrain from using it (Jung et al., 2012; Meuleman et al., 2006). In fact, for clinical approval, FBS is looked as an unreliable supplement for production of a cellular product and its use has been discouraged and should be avoided in accordance with international guidelines and regulatory framework (EMEA, 2008; FDA, 1998; Halme and Kessler, 2006). Additionally, ethical concerns regarding the harvesting and collection of this animal-derived product have been raised (van der Valk et al., 2004).

In light of this, there is a need to find alternative GMP-compliant culture medium formulations that can overcome these drawbacks making the MSC culture a robust, reproducible and safe protocol for clinical translation of the growing field of regenerative medicine. The first challenge is to go from animal-derived products to a xeno-free formulation. Several studies have demonstrated the ability to isolate and expand MSC, in both static and dynamic conditions, using xeno-free formulations or human blood-derived supplements

(Carmelo et al., 2015; Mizukami et al., 2016; Pérez-Ilzarbe et al., 2009; Santos et al., 2011; Soure et al., 2016).

I.8.1. Serum/Xeno-free formulations

The immediate advantage of a serum/xeno-free formulation is the total elimination of both viral and prion contamination, and the lot-to-lot variability. Without the supplementation of serum, the medium remains well defined, where all components are known and at what concentrations, bypassing GMP hurdles of non-defined media. The robustness of a platform using this type of medium increases significantly and can guarantee cell quality.

Although this type of media does not need the supplementation with a serum, there are two types of formulations: one that completely excludes all animal-derived components and a second one that utilizes human-derived proteins so enhance its performance. The first meaning that all components are very well defined and characterized, with all know quantities established, and the latter meaning that although it contains no animal-derived products, human-derived proteins could be used to supplement the medium to obtain a complex variety of growth factors, proteins and hormones. However, this means that this medium will lose the advantage of being well-defined, as the concentrations of added components are not known. An example of this sub-type of media is StemPro® MSC SFM XenoFree culture media from Life Technologies®. Reports have shown the success of this medium to both isolate and expand MSC from BM, UCM and AT under dynamic conditions, while retaining the desired cell characteristics (Carmelo et al., 2015; Simões et al., 2013). Nevertheless, dos Santos and colleagues shown that this formulation expands BM MSC to a further extent than AT MSC, leaving the need to either further understand the nuances of AT MSC or use a more effective medium for this particular cell source (Santos et al., 2011). Furthermore, expanded MSC population were shown similar phenotype, expression profile and differentiation characteristics than ones expanded with serum supplemented media (Chase et al., 2010; Ng et al., 2008).

However, this type of medium requires the addition of a substrate to provide adhesion proteins need for the cells to attach to the culture surface. There are several commercially available substrates can could be used to coat the culture surface, that use human-derived components such as CellStart™ CTS™.

Selecting the appropriate medium with the appropriate supplements proves to be another critical step to ensure efficient expansion of cells. In fact, this demonstrates that there is not one simple and unique solution, but rather making educated choice. Although ideally,

for clinical translation, this type of medium is preferred as all requirements are met for a GMP-grade production of cellular product.

I.8.2. Human AB serum

Another promising option for MSC culture is the substitution of FBS with Human AB Plasma converted into serum, which is routinely tested for viral contamination. The Human AB Serum (hAB) supports the expansion of osteoblasts, chondrocytes and BM cells, as well as glioma and melanoma cells (Bernardo et al., 2007). Recent studies suggest that hAB serum is prime candidate for FBS substitution as medium supplement for cell culture (Cánovas and Bird, 2012; Kocaoemer et al., 2007; Tozetti et al., 2017). Further studies showed that MSC cultured in hAB supplemented medium present a similar growth kinetic, quality and differentiation potential (Bieback et al., 2009).

In order to obtain the human serum, plasma previously separated during blood processing can be readily used, finding it already stocked in blood banks, or blood donation into blood bags without blood thinners (Bieback et al., 2009; Pytlík et al., 2009). Normally, one lot of hAB serum prepared for MSC culture is manufactured from donor pools of at least 5 donors, with the objective to reduce variability between lots. A study performed by Julavijitphong and colleagues showed that hAB serum produced from umbilical cord blood is an effective supplement for the expansion of MSC derived from UCM. However, due to volume constraints, there is a limited yield that can be obtained from each donor (Julavijitphong et al., 2014). The use of hAB serum is still novel and must be standardized in regards of production, assuring the quality and efficacy to promote cellular growth.

MSC cultivated with hAB serum showed increase performance in inhibiting the proliferation of lymphocytes, reaching up to 75% inhibition when combined with different factors (Bartholomew et al., 2002; Fekete et al., 2012; Yamaguchi et al., 2002). Even after extended periods of time, MSC cultivated in hAB serum have shown no genetic alterations, which could compromise their safety. Even karyotype studies were normal after cultivation of cells in allogeneic serum pools. In fact, both allogeneic and autologous usage of hAB serum allowed the expansion of MSC while conversing their desired properties (Bieback et al., 2009).

I.8.3. Human Platelet Lysate

Human platelet lysates (hPL) enriched with growth factors has been presented as a viable alternative as it demonstrated high efficiency and safety when compared to FBS (Schallmoser et al., 2007; Soure et al., 2016). In 1980s, hPL was found to support proliferation of established cell lines and primary fibroblasts. Further analysis of the multiplicity of platelet derived growth factors showed these are involved in vascular regeneration, immune responses and wound healing processes (Hara et al., 1980; King and Buchwald, 1984; Semple et al., 2011).

Recently, hPL was shown to support the expansion of other cells and cell lines, as well as isolation and expansion of MSC. As a matter of fact, MSC exhibit higher proliferative potential when compared with FBS, similar surface markers expression levels and comparable differentiation ability into the three main lineages (Capelli et al., 2007; Horn et al., 2010). Doucet and colleagues showed that medium supplemented with hPL efficiently expands MSC, and since then several reports have shown both allogeneic and autologous hPL to be superior to FBS (Burnouf et al., 2016; Doucet et al., 2005; Soure et al., 2016). The rapid expansion of MSC with this medium enabled a large-scale expansion that could be used in clinical settings, where cells maintained genomic stability (Schallmoser et al., 2008).

Both allogeneic and autologous hPL can be implemented in medical settings for *ex vivo* expansion of MSC. However, similar to hAB serum, autologous hPL has the limitation regarding volume that could be collected, while allogeneic hPL could be pooled and made into an off-the-shelf product (Atashi et al., 2014). The main advantages of using an autologous over allogeneic hPL is avoiding the risk of contamination by virus or prions and immune reactions promoted by internalization of allogeneic proteins by MSC (Mannello and Tonti, 2007). However, this risk might need to be overlooked if the application requires a minimal number of MSC passages when the donor cannot supply with sufficient blood volume. On top of that, the standardization requirement that was mentioned previously is impossible to achieve if autologous option is chosen (Shih and Burnouf, 2015). Nevertheless, allogeneic pooling also increases the risks of contaminations with pathogens.

Oikonomopoulos and colleagues demonstrated that MSC expanded with hPL present reduced immunosuppressive potential, which collides with previous studies that reported the maintenance of immunomodulatory properties (Capelli et al., 2007; Doucet et al., 2005; Oikonomopoulos et al., 2015). These contradictory data might result from the inconsistency of hPL production.

hPL is produced by repeated freeze/thaw cycles and sonication from fresh blood or platelet concentrates, containing various bioactive molecules such as chemokines, growth factors and adhesion molecules. Recently, hPL has been considered a potential gold standard supplement for tissue engineering and regenerative medicine (Burnouf et al., 2016). However, considering the need for pool allogeneic hPL for clinical applications, industry has made available several large pool hPL products, but there still exists a huge disparities on the methodologies for production (Bernardo et al., 2007; Bieback et al., 2009; Salvade et al., 2009; Schallmoser et al., 2007). The hPL formulations are rapidly evolving, as for example early formulations had incomplete fibrinogen depletion, which required the addition of animal-derived heparin. Moreover, hPL can be virally inactivated by pasteurization, nanofiltration dry heat, UV and solvent/detergent incubation, among others, in order to increase product safety (Burnouf and Radosevich, 2000).

The use of hPL as supplement for expansion of cells for transplantation brings into question if the supply will be sufficient considering the increasing demands of cellular products.

I.9. Aim of Studies

With the increase interest in the last decade of the regenerative and immunomodulatory properties of MSC, the number of clinical trials worldwide for a wide variety of diseases (graft-versus-host disease, myocardial infarction or Crohn's disease, among others) has been growing. However, traditional 2-D culture methods are very labor intensive and limited in scalability, stressing the need to a large-scale platform for MSC cultivation that meets the clinical and commercial relevant lot sizes.

The main goal of this project is to establish a platform the scalable production of human MSC in a 3-D suspension culture under a xenogeneic-free condition. Two difference approaches were used for MSC cultivation: spheroids and microcarriers. Specifically, this thesis has the following objectives:

1. Optimization of spheroid culture conditions from three different sources: BM, AT and UCM;
2. In vitro characterization of human MSC cultured as spheroids regarding spheroid size, density, MSC source, media formulation and dynamic conditions;
3. Selecting ideal microcarrier for MSC culture support;
4. Optimization of dynamic culture conditions, giving special attention to xeno-free media
5. Scale-up cultivation of human MSC to a stirred tank reactor for large-scale production of cells

Chapter II

Establishing culture conditions for human mesenchymal stem/stromal cells cultivation as 3-D spheroids, under xeno-free conditions

Chapter II - Establishing culture conditions for human mesenchymal stem cells cultivation as 3-D spheroids, under xeno-free conditions

II. Introduction

The implementation of mesenchymal stem/stromal cells in regenerative medicine is still critically hindered by the lack of a robust platform that allow efficient and economical viable expansion that can be reproduced with ease (Caplan, 2007).

Despite the majority of reports concerning culture of MSC refer to static monolayer cultures, academia is looking at dynamic 3D cultures that could more precisely mimic *in vivo* conditions, and also allow scale-up. 3D aggregates, referred to as spheroids, can mimic more precisely cellular adhesion and signalling that MSC present in their *in vivo* niches (Saleh and Genever, 2011). In addition, the therapeutic potential of cells cultured as spheroids have shown to have higher expression of adhesion molecules and anti-inflammatory proteins (Bartosh et al., 2010). Moreover, considering that clinical applications would require the infusion of MSC into patients, the cell entrapment in the lungs is one of the major obstacles which could be overcome due the considerably smaller diameter of cells released from spheroids when compared to monolayer cultures (Baksh et al., 2007). Regarding the most common used 3D support system for adherent cells expansion, microcarriers, spheroids present the advantage of not needing an external non-biological component (e.g. as scaffold) that must be retrieved in the downstream process prior to any clinical application.

The hanging drop method used for spheroid formation prior to its cultivations present poor translation to clinical settings, as spheroid size is difficult to control and the method of culture is ineffective to expand cells in this conformation (Frith et al., 2009). Hence, there is the need for a robust, GMP-compliant and reproducible bioprocess for expansion of MSC culture as spheroids.

II.1. Material and methods

II.1.1. Mesenchymal stem/stromal cell sources and isolation

For this work, human MSC were isolated from three different sources: BM, UCM and AT. BM samples were obtained from healthy donors at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal. The UC were obtained from Hospital São Francisco Xavier, Lisboa. AT samples 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.

MSC, kept cryopreserved in the lab, were thawed and used according to need. specific cell passage was considered, as this was a considered a preliminary study, however cells were not culture past passage 5.

II.1.1.1. Bone marrow mesenchymal stem/stromal cells isolation

Bone marrow isolation was not performed for this work. However, the cells used throughout this thesis were isolated as described by a study performed by dos Santos and colleagues (dos Santos et al., 2010).

II.1.1.2. Umbilical cord mesenchymal stem/stromal cells isolation

Arteries and vein are dissected from UCM, and Wharton's Jelly is further minced into small fragments. For isolation with 10% FBS supplemented media, these fragments are then mixed with Collagenase type II (Sigma-Aldrich®) and PBS (Gibco®). The enzymatic digestion takes up to 4 hours at 37ºC with along with agitation set at 700 rpm. After incubation, suespension was filtered and centrifuged several times. Pellet was resuspended in culture medium and cells were counted under optical microscope using Turk's Reagent Solution (Merck Millipore). Cells were then plated on T-flasks (Falcon BD Biocience®) with a density of 10 000 cells/cm^2. For isolation with hPL supplemented medium or xeno-free culture medium, the fragments were plated as explants on six-well plates (Corning Inc.) with either low-glucose DMEM supplemented with 5% (v/v) UltraGro™ (AventaCell BioMedical) containing 2 IU/ml of heparin (Sigma-Aldrich®), or StemPro® MSC SFM XenoFree (Life Technologies). For the latter culture medium, the six-well plates were previously coated with

CELLstart™ CTS™ (Life Teachnologies). All solution used contained 1% Antibiotic-Antimycotic (A/A) (Life Technologies).

II.1.1.3. Adipose tissue mesenchymal stem/stromal cells isolation

25 to 200 ml of AT aspirates were washed with PBS with 1% A/A. Two different approaches were used for further isolation, one using collagenase type II and another using stronger mechanical agitation in order to break ECM. For FBS supplemented media and StemPro® MSC SFM XenoFree the isolation was performed using collagenase, and for Ultragro™ the non-enzymatic method was performed. The solution was either agitated gently our vigorously for enzymatic and non-enzymatic method, respectively. After a short resting period, a phase separation was observed, and the supernatant was removed and a new washing cycles was initiated. This step was repeated 2-3 times until solution presented a clearer yellow colour indicating the removal of erythrocytes. The remaining infranatant, for the enzymatic method, was collected and incubated with 0.1% of collagenase type II (Gibco®) in Hank's Buffered Salt Solution (Gibco®) for 30 minutes at 37ºC, or, for a non-enzymatic method, resuspended and centrifuged in culture media. Enzymatic digestion required an additional step of filtration using Steriflip filter unit (Millipore) and then centrifuged. The pellet, the stromal vascular fraction, of both methods was then collected and one more centrifuged before the number of cells and their viability was assessed using Turk's Reagent Solution (Merck Millipore). Cells were then plated at a density of 100 000 cells/cm^2 in order to isolate AT MSC based on their adherence to the plastic surface. The medium used was low glucose DMEM with 10% FBS for the enzymatic method, and for the non-enzymatic, with either low-glucose DMEM supplemented with 5% (v/v) UltraGro™ (containing 2 IU/ml of heparin (Sigma-Aldrich®), or StemPro® MSC SFM XenoFree. For the latter culture medium, the six-well plates were previously coated with CELLstart™ CTS™.

II.1.2. Culture media

MSC were cultured using four different medium formulations: DMEM supplemented with 10% FBS, DMEM supplemented with 5% hPL, StemPro® MSC SFM XenoFree and Corning™ StemGro™. All media were supplemented with 1% Antibiotic-Antimycotic (A/A) (Life Technologies). For 2D cultures, when using serum-free media (StemPro® MSC SFM

XenoFree and Corning™ StemGro™) the surface used for cell culture was coated with CELLstart™ CTS™.

II.1.3. 2D static culture

After thawing, cell number and viability were assessed using trypan blue (Gibco®) exclusion method. Cells were then plated at a 3 000 cell/cm^2 on T-flasks. These were cultured inside a humidified incubator at 37ºC with 5% CO_2, with culture medium exchanged every 3-4 days. Upon reaching approximately 80% confluence, exhausted medium was removed, and cells were washed with PBS solution and were passaged using 1% TrypLE solution (Life Technologies) or Accutase (Sigma-Aldrich®) for xeno-free media, or 0.1% Trypsin (Life Technologies) for FBS containing medium. After each passage, cells were counted using the trypan blue exclusion method. For cultures using StemPro® MSC SFM XenoFree medium a pre-coating of the tissue culture flasks with CELLstart™CTS™ was performed upon thawing and at each passage.

II.1.4. 3D culture

II.1.4.1. Spheroid formation

MSC were aggregated into spheroids using the AggreWell™ plates (STEMCELL Technologies™). These plates needed to be primed before MSC aggregation protocol, where the plate was rinsed with 2 ml of PBS solution and then aspirated. Culture medium was then added, 0.5 ml, and the plate was centrifuged at 3500 rpm for 5 minutes in order to remove air bubbles from the microwells. After careful observation under the microscope, if the plates retained air bubbles the washing process had to be performed again.

After priming, cells were resuspended in the determined density for a 1.5 ml volume that was added to each well of the plate. Then, a gently pipetting was performed several times to evenly distribute cells throughout every microwell. Plate was centrifuged at 1500 rpm for 10 minutes to entrap cells in the bottom of each microwell. Afterwards, the plate was once again carefully observed under microscope to assure no air bubbles remained and that cells were evenly distributed among the microwells. The plate was then placed inside the incubator, and spheroid formation was attained by a 24h period incubation.

For spheroid formation, two different commercially available AggreWell™ plates were used: AggreWell™ 400 and AggreWell™ 800. Both are 24-well-like plates that have 8 non-

reusable wells. AggreWell™ 400 contains approximately 1200 microwells per well, while AggreWell™ 800 contains approximately 300 microwells per well. Although optimized for embryonic stem cells and induced pluripotent stem cells, according to manufacturer guidelines, AggreWell™ 400 is optimal for the formation of spheroids from 50 to 3000 cells per spheroid, while AggreWell™ 800 is optimal for spheroids containing between 3000 to 20 000 cells. However, for MSC, AggreWell™ 800 was used for the formation of spheroids with or higher than 1000 cells per spheroid. Figure 6 shows images of Aggrewells™ with different densities of cells and how cells are forced into the bottom of the microwells promoting aggregation.

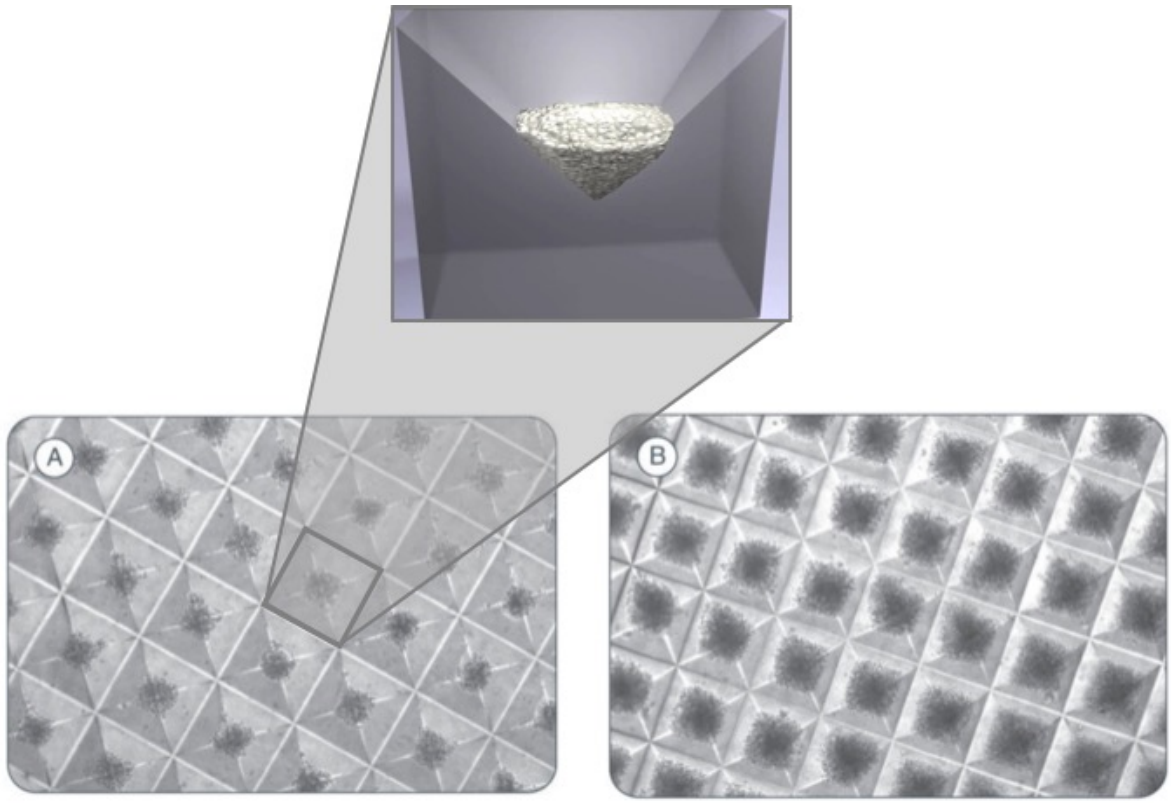

Figure 6 – Schematic representation of Aggrewells™. **(A)** and **(B)** show different densities of spheroids being formed in the different sized wells.

II.1.4.2. Filtration of Spheroid suspension

After spheroid formation, not all cells inserted in the AggreWell™ aggregated. Some were dead or simply could not aggregate into spheroids, remaining in suspension. In order to remove such cells or cellular debris, spheroid suspension was filtered, and non-aggregated cells counted using Trypan Blue exclusion method. Spheroids were harvested by gently pipetting the medium inside each well with a 1 ml tip. The medium is passed through a 37 μm reversible cell strainer (STEMCELL Technologies™) allowing the passage of single-cells and cellular

debris. Afterwards, the strainer is reversed and the aggregates that were retained in the filter are eluted using the culture medium.

By subtracting the single-cells filtered to the number of cells used in the AggreWell™, and assuming even distribution, the number of cells per spheroid can be estimated.

II.1.5. Spheroid culture

II.1.5.1. Culture supports

As MSC are adherent cells, special design supports were needed to prevent cells to migrate from spheroids onto the plastic surface. Hence, MSC spheroids were cultured on ultra-low attachment 6-well plates (Costar® Corning®), ultra-low attachment 96-well plates (Thermo Fisher®), 100 mm bacteriologic grade Petri dishes or spinner flasks (Belco®).

II.1.5.2. Dynamic conditions

All cultures were performed inside a humidified incubator set at 37ºC with 5% CO_2. Dynamic conditions were achieved by using a rotary orbital shaker, Rotamax 120 (Heidolph). Agitation speed used was 65 rpm.

II.1.5.3. Spheroid dissociation

Using the reversible strainer, spheroids were collected and transferred into a round bottom falcon tube and centrifuged for 7 minutes at 1250 rpm. After centrifugation, about 90% of medium was removed with a pipette and PBS was added to wash the spheroids. After resuspension, spheroids were centrifuged again and about 90% of the supernatant was removed. For enzymatic dissociation 0.1% trypsin solution was added. The spheroid suspension was then incubated at 37°C, 700 rpm for 7-15 minutes in a Thermomixer (Eppendorf®). After incubation with trypsin, culture medium was added in a proportion of 4 times the volume of trypsin solution, and the mixture centrifuged for 10 minutes and 1750 rpm.

The supernatant was then discarded, cells resuspended in medium and counted using the Trypan Blue exclusion method.

II.1.6. Spheroid characterization

II.1.6.1. Cell proliferation

For the determination of cell expansion within spheroids, after dissociation, cells were counted using the using the Trypan Blue exclusion method. Following this procedure for each time point of interest, cell fold expansion was determined using the following equation:

$$Cell\ Fold\ = \frac{Number\ of\ cells\ at\ day\ X}{Number\ of\ cells\ at\ day\ 1},$$

where x is the time point select for assessment of spheroid proliferation.

Cell proliferation was also accessed using KI-67. The staining of MSC with KI-67 obtained from spheroids were performed by fixing cells with 2% paraformaldehyde (PFA) overnight. The next step consists of coating eppendorf tubes with 1% bovine serum albumin (BSA) for 15 minutes. Meanwhile, the samples of cell suspension previously stored in PFA were centrifuged at 1250 rpm for 7 minutes. After centrifugation, cells were washed twice with 1% normal goat serum (NGS - Sigma®). Another centrifugation step was performed after each washing step. Then, cells were resuspended in 3% NGS. Cells were centrifuged at 1250 rpm for 3 minutes and then permeabilized with a solution 1:1 of 3% NGS and 1% saponin for 15 minutes. Washing with 3% NGS and centrifugation steps were required. After this, cells were resuspended in primary antibody solution Ki-67 and incubate for 90 minutes in the dark, at room temperature. Washing with 3% NGS and centrifugation steps were repeated twice. Cells were resuspended in secondary antibody solution, including the negative control. After 45 minutes, cells were washed with 1% NGS, resuspended in PBS. Cells were analyzed by flow cytometer, with FACSCalibur™, and under a fluorescent microscope.

II.1.6.2. Immunophenotype characterization

Cells are centrifuged at 1250 rpm for 7 min, the supernatant is discarded, and cells are resuspended in PBS to obtain 100 μL of cell suspension per FACS tube. To each FACS tube 5 μL of the specific antibody is added, and incubated in the dark at room temperature for 15 min.

After the incubation, 2 ml of PBS are added to each FACS tube and the solution is centrifuged for 5 min at 1000 rpm. Supernatant is removed, and cells are resuspended in the remaining PBS solution. 500 μL of 1% PFA are added to each tube and kept stored at 4ºC until analysis. Using a panel of mouse anti-human monoclonal antibodies, MSC expanded under static or dynamic conditions were analysed by flow cytometry for the expression of CD90, CD73, CD105, CD34, CD14, CD19, CD31, CD45, CD80, HLA-DR, and CD54. Isotype controls were also prepared and a minimum of 10000 events are acquired for each sample. Samples were analysed in a FACScalibur (Becton Dickinson) flow cytometer and CellQuest™ software (Becton Dickinson) was used for acquisition. Analysis was performed using the FlowJo software (Tree Star).

II.1.6.3. Diameter of the spheroids

Multiple photographs were taken using an optical microscope (Leica Microsystems, Carnaxide, Portugal) for every time point of interest. Spheroid diameters were then measured using ImageJ software. Considering the spherical-like conformation of spheroids, a script was written that would approximate the outlined borders to a sphere and then diameter was calculated. The number of spheroids used for diameter determination varied between experiments, depending on the availability of these, but no $n < 60$ was used.

II.1.6.4. Statistical analysis

Statistical analysis is presented as mean + standard deviation (SD). Two-way ANOVA was calculated between different experimental groups and Tukey's multiple comparisons test was performed to determine statistically significant differences ($p < 0.05$).

II.2. Results and discussion

II.2.1. Spheroid formation

Although AggreWell™ is optimized for spheroid formation of embryonic stem cells and induced pluripotent stem cells, this protocol was used in this work for the reproducible generation of spheroids with human MSC. Three different densities were formed using this method: 100 cells per spheroid (cell/sph), 500 cells/sph and 1000 cells/sph. To determine the viability of this method to produce spheroids with the number of cells desired, several tests were used.

First, it was determined the number of cells that were filtered after spheroid formation (see section II.1.4.2). With the intent to generate 1000 cells/sph, $3x10^5$ cells were added to each well of the plate, as this Aggrewell™ has 300 wells. After the formation process and filtration, both single-cell and spheroids were counted. In fact, assuming the even distribution of cells amongst each spheroid, each spheroid would have 964 ± 10 cells. For the 100 cell/sph, 89 ± 6 cells were counted. This means that the spheroid formation has an error of approximately 4% and 11%, respectively.

Secondly, in order to determine if the cell distribution was effective between microwells hence leading to reproducible formation of 1000 cell/sph, a test was performed where spheroid culture was successively diluted until reach a single spheroid per count (n = 50). In this test, similar results were obtained, 959 ± 16 cells and 91 ± 11 cells per spheroid. The standard deviation is higher due to the practical challenge in counting such a lower number of cells. In addition, spheroid dissociation is a highly "harsh" process, which might lead to an increase in difference between cell number obtained and expected. For these tests, a monolayer cell expansion and a spheroid formation protocol using DMEM supplemented with 10% FBS was employed.

II.2.2. Static culture

II.2.2.1. AT, BM and UCM MSC spheroids

With the robustness of the process established, the aim of this work was to promote expansion of MSC in the spheroid configuration. Three different cell sources were used in two different cell densities (100 cells/sph and 1000 cells/sph) and in two different culture media (DMEM + 10% FBS and StemPro® MSC SFM XenoFree). Spheroids were cultured for a 6 days period and evaluated at the end of culture for both spheroid diameter and number of cells.

For 10% FBS supplemented medium, no significant differences were observed between sources, Figure 7. Spheroids with cells from the three sources had similar diameters on both densities and the fold increase in cell number obtained was similar between them. On day 1, the first day after spheroid formation, spheroids of 100 cells/sph had a diameter of 65.4 ± 12.2

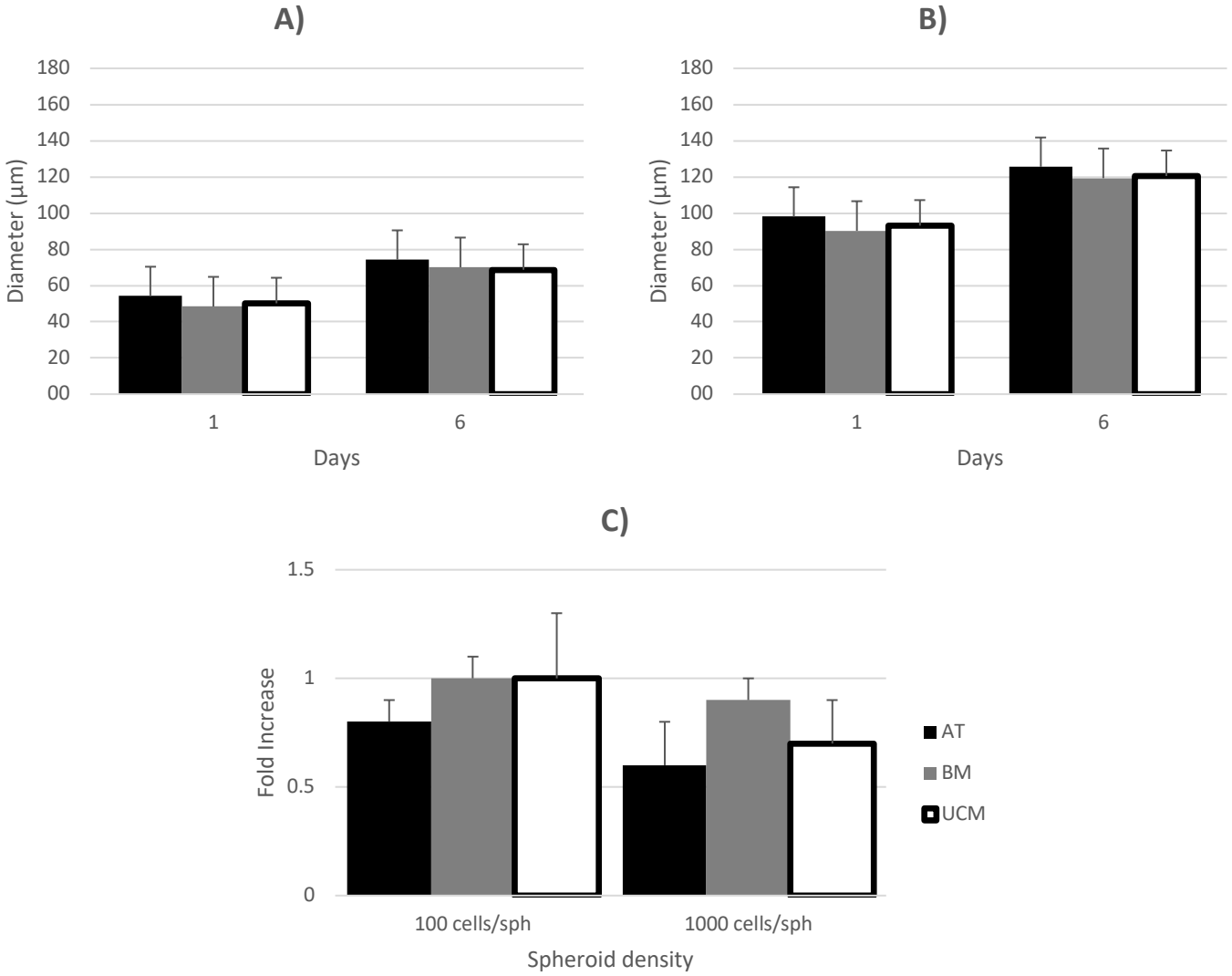

Figure 7 - Diameter of spheroids initiated with 100 cells/sph (**A**) and 1000 cells/sph (**B**) for AT MSC (black), BM MSC (grey) and UCM MSC (white) in the first day and at day 6, when cultured in StemPro MSC SFM XenoFree. For day 1 and day 6 respectively: AT MSC 100 sph/cells – 54.2 ± 13.4 µm and 74.3 ± 16.2µm; AT MSC 1000 sph/cells – 98.1 ± 11.6 µm and 125.6 ±15.2 µm; BM MSC 100 sph/cell – 48.4 ± 12.7 µm and 70.1 ± 16.4 µm ; BM MSC 1000 cells/sph – 90.2 ± 9.8 µm and 119.3 ± 12.4 µm; UCM MSC 100 cells/sph – 50.2 ± 10.5 µm and 68.7 ± 14.1 µm; UCM MSC 1000 cells/sph – 93.1 ± 12.3 µm and 120.5 ± 15.9 µm. **(C)** Fold increase determined after the end of culture for each source and cell density. AT MSC 100 sph/cells – 0.8 ± 0.1; AT MSC 1000 sph/cells – 0.6 ± 0.2; BM MSC 100 sph/cell – 1.0 ± 0.3; BM MSC 1000 cells/sph – 0.9 ± 0.1; UCM MSC 100 cells/sph – 1.0 ± 0.3; UCM MSC 1000 cells/sph – 0.7 ± 0.2. (n = 3)

μm, 63.7 ± 14.8 μm and 59.3 ± 9.5 μm for AT, BM and UCM cells, respectively. After 5 days in culture, at day 6, these had 77.4 ± 17.1μm, 73.5 ± 18.4 μm and 63.4 ± 13,4 μm for AT, BM and UCM, respectively. Although not statistically significant, except for the AT MSC 1000 cell/sph ($p = 0.03$), the spheroids seem to increase in size when in culture. The same phenomena were observed (still not being statistically significant) for spheroids with 1000 cells/sph. AT MSC spheroids at day 1 had 104.1 ± 11.6 μm whereas at day 6 presented 145.3 ±15.2 μm. The same way for BM and UCM cells at day 1 had 110.3 ± 10.2 μm and 106.5 ± 14.6 μm, and at day 6 136.2 ± 13.4 μm and 130.9 ± 14.8 μm, respectively. However, this apparent increase in size is not due to the expansion of cells as the fold increase values obtained do not show cell proliferation. In fact, the higher fold increase value observed was for spheroid from BM MSC with 1.2 ± 0.1. Which means that there was little to no expansion of MSC in this 3D configuration, despite the apparent increase in size. One possible explanation for this observation would be that the level of compactness of the spheroid would diminish after its formation.

Furthermore, results from StemPro® MSC SFM XenoFree exhibits the same patterns, as presented in Figure 8. No significant differences were observed between cells from different sources, and an apparent, although not statistically significant, increase in size at the end of the culture. However, when comparing both media, a decrease in overall size of the spheroid seems to occur. As discussed in section I.8, culture medium has influence in various aspects of cells,

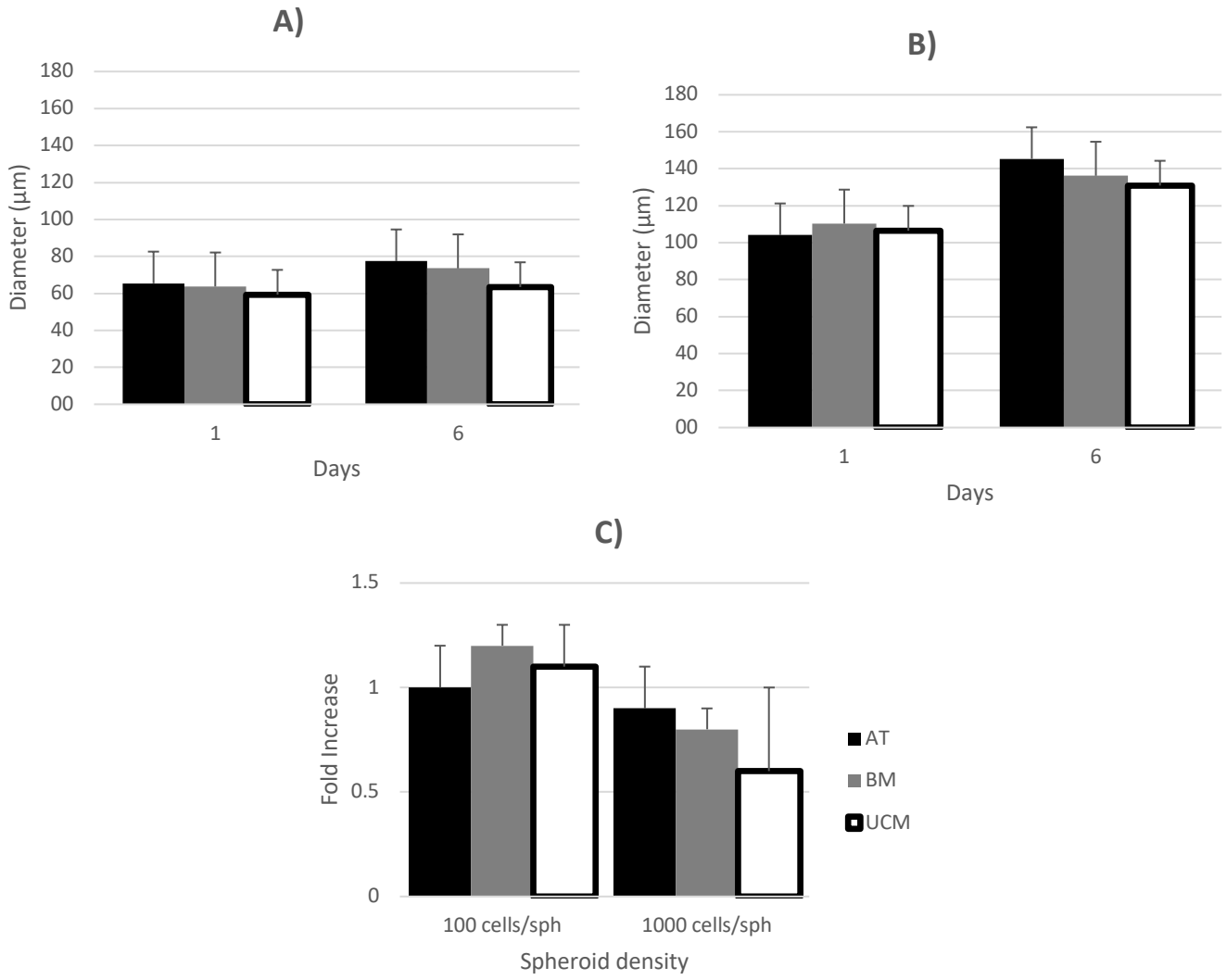

Figure 8 - Diameter of spheroids initiated with 100 cells/sph (**A**) and 1000 cells/sph (**B**) for AT MSC (black), BM MSC (grey) and UCM MSC (white) in the first day and at day 6, when cultured in DMEM supplemented with FBS. For day 1 and day 6 respectively: AT MSC 100 sph/cells – 65.4 ± 12.2 µm and 77.4 ± 17.1µm; AT MSC 1000 sph/cells – 104.1 ± 11.6 µm and 145.3 ±15.2 µm; BM MSC 100 sph/cell – 63.7 ± 14.8 µm and 73.5 ± 18.4 µm ; BM MSC 1000 cells/sph – 110.3 ± 10.2 µm and 136.2 ± 13.4 µm; UCM MSC 100 cells/sph – 59.3 ± 9.5 µm and 63.4 ± 13,4 µm; UCM MSC 1000 cells/sph – 106.5 ± 14.6 µm and 130.9 ± 14.8 µm. **(C)** Fold increase determined after the end of culture for each source and cell density. AT MSC 100 sph/cells – 1 ± 0.2; AT MSC 1000 sph/cells – 0.9 ± 0.2; BM MSC 100 sph/cell – 1.2 ± 0.1; BM MSC 1000 cells/sph – 0.8 ± 0.1; UCM MSC 100 cells/sph – 1.1 ± 0.2; UCM MSC 1000 cells/sph – 0.6 ± 0.4. (n = 3)

and selecting the appropriate medium might be another parameter to further conditioning of spheroid size. In fact, Riis and colleagues showed that different media could influence cell size, which in turn could allow us to control spheroid size (Riis et al., 2016).

Another observation would be that 100 cells/sph cultured in DMEM supplemented with 10% FBS present a slightly higher fold increase in cell number when compared to 1000 cells/sph. This observation is further supported by the StemPro® MSC SFM XenoFree experiment, where the fold increase value was once again influenced by spheroid density. This observation can be justified by nutrient and oxygen gradients formed within the spheroids, which may limit the availability of nutrients and oxygen to cells closer to the core of the spheroid. A diameter higher than 150 μm has been reported to hinder the oxygen and nutrient diffusion throughout the spheroid (Saleh and Genever, 2011). With this in mind, an experiment was performed, section II.2.2.2, in order to determine the optimal spheroid size to be used.

Additionally, no significant difference was observed between the two media regarding fold increase in total cell number. Although slightly higher with FBS supplemented medium, fold increase values were still low using either media formulation. However, Alimperti and co-workers were able to reach a 4-fold increase in number of cells, where a Design of Experiments methodology was used. They were able to prepare a media formulation, which was not disclosed, that consisted in a combination of two media (50/50) optimal for suspension culture of spheroids (Stella et al., 2014). This report shows that if we used more specific and optimized formulation for spheroid culture, the results might have been different. Further tests were performed using FBS supplemented media until stated otherwise.

II.2.2.2. Number of cells per spheroid

Regarding the diffusional problems, an experiment was performed to define an optimal size of cell/sph with AT MSC and UCM MSC. Three different densities were tested: 100 cells/sph, 500 cells/sph and 1000 cells/sph, Figure 9. The same 6-day culture period was performed, and spheroid diameter was determined, calculated as well as fold increase in cell number. The same tendency was observed, where spheroids showed an increase level of compactness characteristic of the spheroid formation. More importantly, for the three densities tested, only 100 cell/sph and 500 cell/sph retained a diameter inferior than 150 μm. At day 6 of culture, for AT and UCM MSC, respectively, spheroids initiated with 100 cells/sph had 7.4 ± 17.1 μm and 63.4 ± 13.4 μm, and 500 cells/sph spheroids had 137.2 ± 14.2 μm and 139.2 ± 12.6 μm. When comparing these two densities regarding fold increase values, although not statistically different, 500 cells/sph spheroids appears to be slightly higher. Interestingly, for

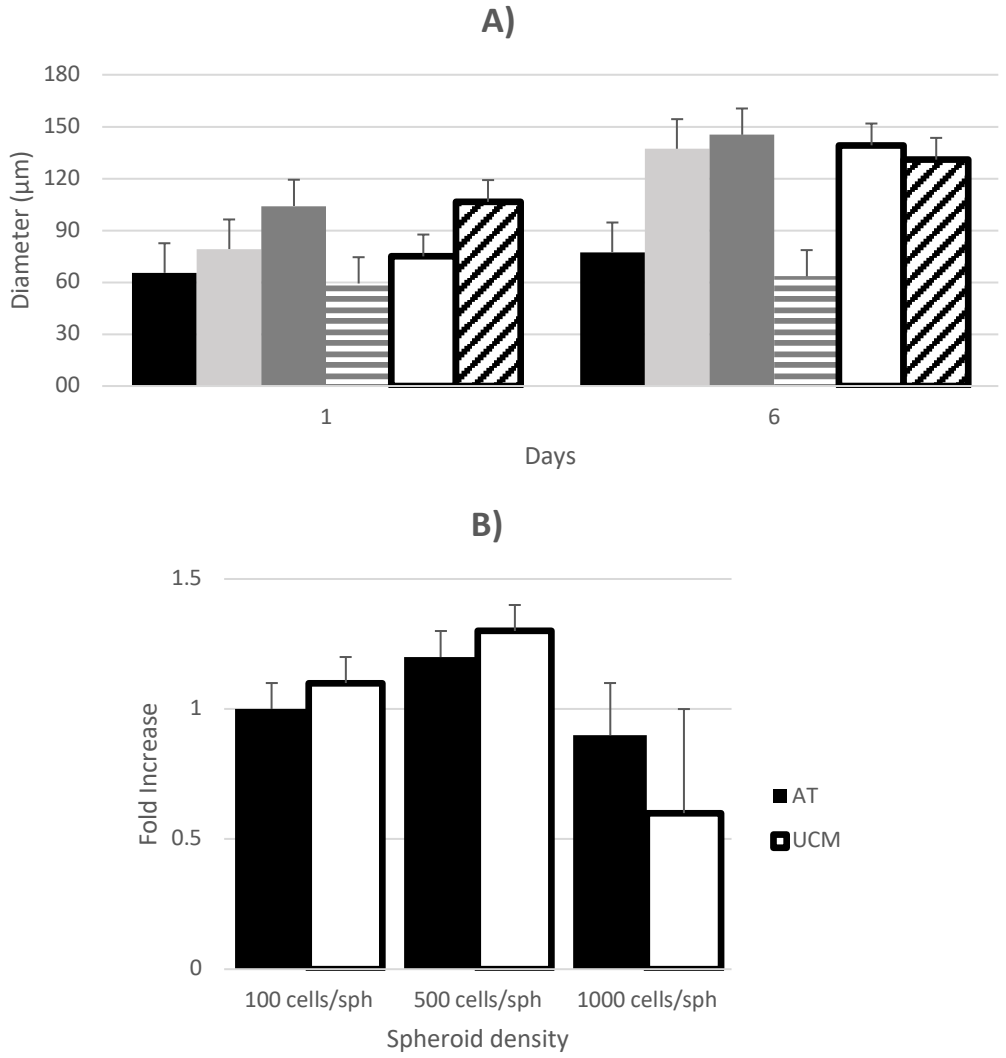

Figure 9 – (A) Diameter of spheroids initiated with AT MSC 100 cells/sph (black), AT MSC 500 cells/sph (light grey), AT MSC 1000 cells/sph (dark grey), UCM MSC 100 clls/sph (horizontal pattern) UCM MSC 500 cells/sph (white) and UCM MSC 1000 cells/sph (diagonal pattern) in the first day and at day 6. For day 1 and day 6 respectively: AT MSC 100 sph/cells – 65.4 ± 12.2 µm and 77.4 ± 17.1 µm; AT MSC 500 sph/cells – 79.2 ± 11.6 µm and 137.2 ±14.2 µm; AT MSC 1000 sph/cells – 104.1 ± 11.6 µm and 145.3 ± 15.2 µm ; UCM MSC 100 cells/sph –59.3 ± 9.5 µm and 75 ± 11.3 µm; UCM MSC 500 cells/sph – 75.0 ± 11.3 µm and 139.2 ± 12.6 µm; UCM MSC 1000 cells/sph – 106.5.1 ± 14.6 µm and 165.2 ± 14.8 µm. **(B)** Fold increase in cell number determined after the end of culture for each source and cell density. AT MSC 100 sph/cells – 1.0 ± 0.1; AT MSC 500 sph/cells – 1.2 ± 0.1; AT MSC 1000 sph/cell – 0.9 ± 0.2; UCM MSC 100 cells/sph – 1.1 ± 0.1; UCM MSC 500 cells/sph – 1.3 ± 0.1; UCM MSC 1000 cells/sph – 0.6 ± 0.4. (n = 3)

the 500 cells/sph density, the increase in size of the spheroid with regard to day 6 of culture, is statistically significant ($p<0.05$) for both AT and UCM MSC. For these reasons, the density 500 cells/sph was chosen to pursuit further studies.

II.2.2.3. Combination of different sources of MSC within spheroids to support cell expansion

Considering that cells from different sources present different properties in regard to ECM production, growth factors secreted and even proliferative capacity, a strategy comprising the combination of different sources was developed (Klingemann et al., 2008).

Spheroids were formed from each cell source and were cultivated in co-culture. All combinations of the three sources were tested and results are displayed in Figure 10, where experiments with AT with BM, AT with UCM, BM with UCM cells and the three combined are shown. When compared to single cell source spheroid culture, the fold increase in cell number obtained was not higher, with AT and BM derived spheroids having 1.2 ± 0.3, AT and UCM derived having 1 ± 0.2, BM and UCM derived 0.9 ± 0.3 and the combination of all three sources 0.6 ± 0.2. Overall, no significant proliferation was observed within spheroids.

At the same time, cultures were expanded to 14 days instead of the 6 days established, with the objective of verifying that it was not an atypical delayed lag phase, where cells needed to adapt to the culture conditions namely the 3D culture format. However, only further aggregation of spheroids was observed, with fold increase maintaining the same levels.

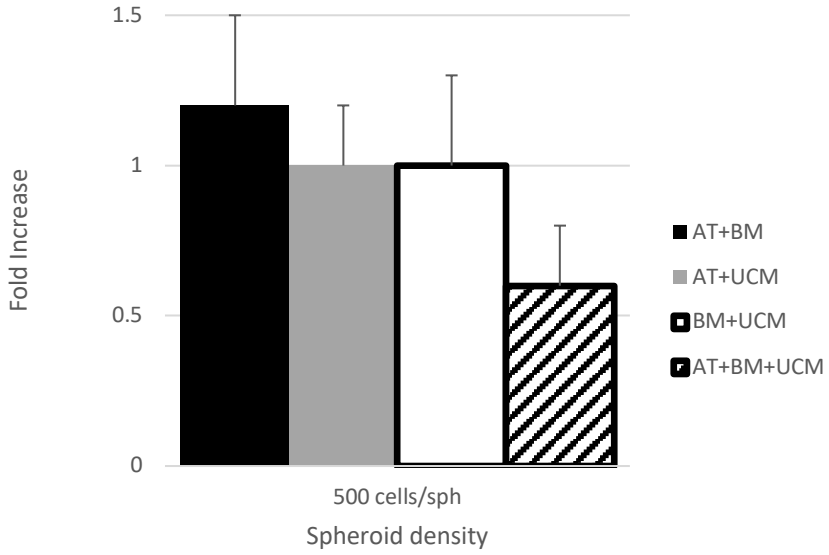

Figure 10 – Fold increase in cell number determined for spheroid culture of multiple cell sources after 6 days of culture. AT and BM MSC (black) - 1.2 ± 0.3; AT and UCM MSC (grey) - 1 ± 0.2; BM and UCM MSC – 0.9 ± 0.3; AT, BM and UCM MSC (pattern) - 0.6 ± 0.2. (n = 2)

II.2.3. Dynamic culture

One observation that was consistent throughout all the experiments, was the aggregation of spheroids throughout culture. As longer the culture continued, less isolated spheroids were observed and more massive deformed structures were visible, Figure 11. These structures were present in all conditions despite spheroid size, source or medium used. In an attempt to limit the spheroid aggregation, agitation was introduced in the system, decreasing the amount of time spheroids would dwell near each other. This control over spheroid aggregation is of extreme importance due to the ability to control spheroid size and minimize diffusion gradients of nutrients and oxygen. Moreover, dynamic systems allow the washout of waste products and/or the broadcast of growth factors that could enhance cell proliferation.

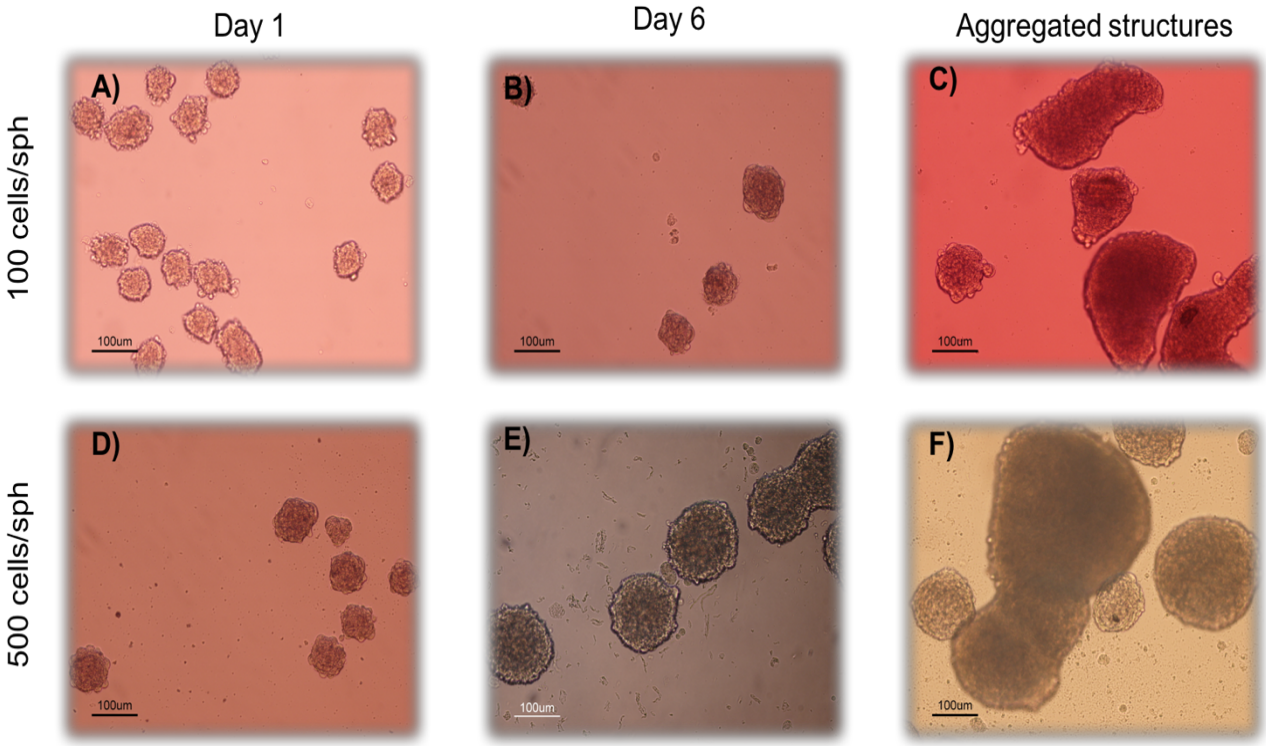

Figure 11 – Spheroids of different spheroids densities at day 1 and day 6. **(A, B and C)** – 100 cells/sph cultured; **(D, E and F)** – 500 cells/sph culture; **(C and F) –** deformed 3D structures generated by the aggregation of several spheroids, increasingly more common as culture period increases **(A,B,C and D)** were cultured in DMEM supplemented with FBS; **(E and F)** were cultured in StemPro® MSC SFM XenoFree.

II.2.3.1. Agitation

Using a rotary orbital shaker three set points of agitation were tested: 20 rpm, 65 rpm and 125 rpm, Figure 12. The lowest agitation proven not to be sufficient to eliminate the spheroid aggregation obstacle and did not have any visible impact in the culture. On the other hand, 125 rpm proved to be too harsh for spheroids as the collision between them disrupted the spheroids and none remained after 2-3 days of culture. Despite the favourable conditions that inhibit spheroid aggregation and continuous medium flow, cell proliferation was not enhanced, with fold increase values of 1.1 ± 0.2. However, one noteworthy observation was the significant decrease in standard deviation regarding spheroid diameter, 110.4 ± 5.6 μm. In fact, this is a characteristic maintained throughout the following experiments while using 65 rpm agitation, suggesting an increase in robustness concerning the spheroid cultivation protocol, either due to preventing formation of spheroid aggregation or to collision between spheroids, eliminating the loosened spheroids.

Figure 12 - Rotamax 120 from Heidolph®

II.2.3.2. Effect of conditioned culture medium on cell proliferation within spheroids

As it was mentioned previously, culture medium plays a major role in cell proliferation, providing not only the nutrients needed but also a cocktail of hormones, growth factors, adhesion proteins and other proteins paramount for cell growth. In light of this, two different strategies were devised to try to improve cell proliferation within the spheroid.

The first method was to use conditioned medium from monolayer cultured cells to supplement spheroid cultures, in order to already include the secreted factors and cues that would lead to cell expansion. Culture media were retrieved from monolayer cultures (T-flasks) of AT, BM and UCM MSC, and added to spheroid culture every 2 days to safeguard for nutrient

depletion or by-product accumulation (Figure 13A). However, by analysing the data, no significant improvement was obtained in regard to cell proliferation.

The second approach was to culture spheroids in different culture media. UCM MSC derived spheroids were cultured using 4 different media for comparison: DMEM supplemented with 10% FBS, StemPro® MSC SFM XenoFree (commercially available), DMEM + 5% hPL and Corning® StemGro® (also commercially available) (Figure 13B and C). The medium effect on the diameter is not statistically evident but, in for the conditions of the study, the

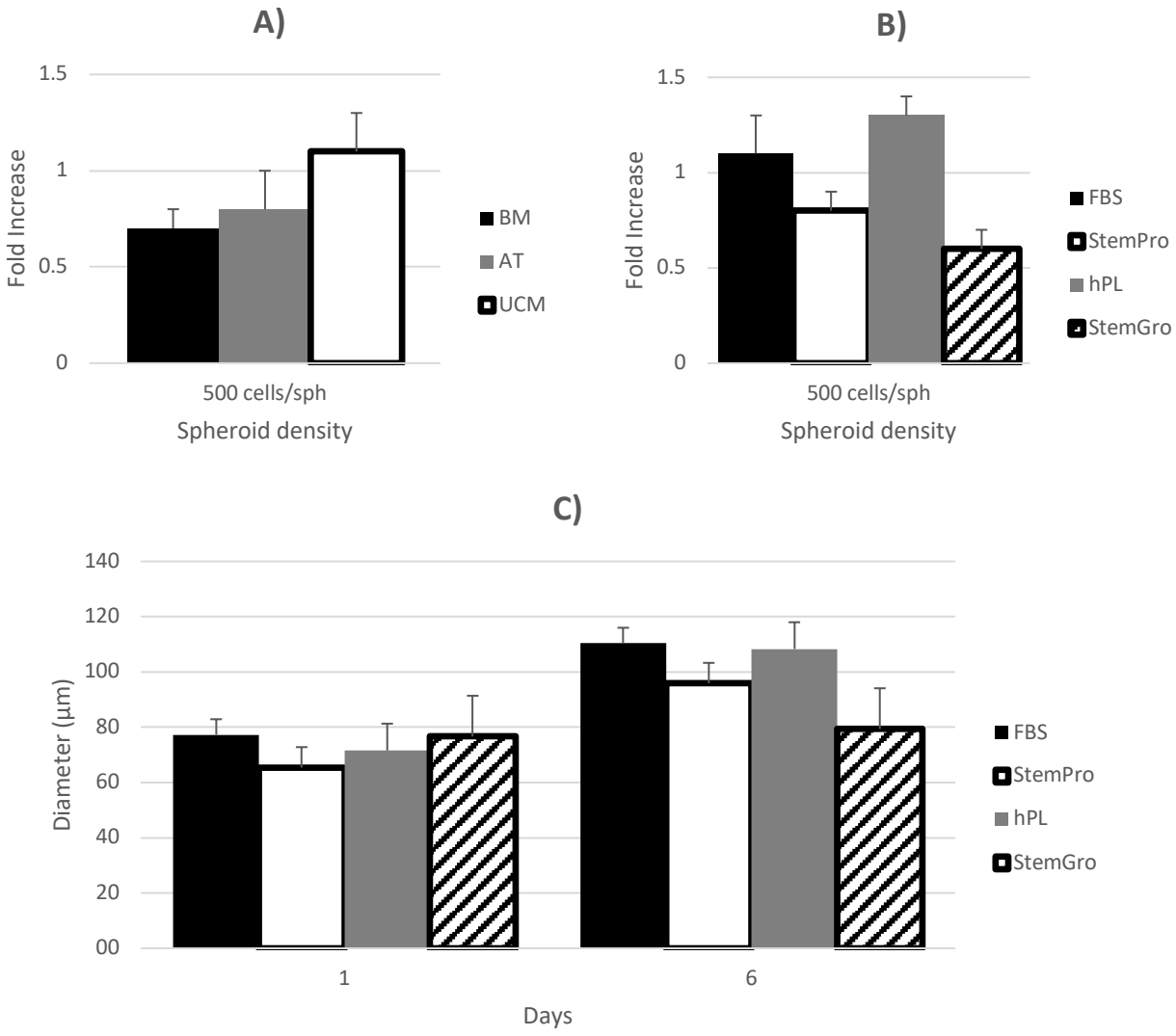

Figure 13 – **(A)** Fold increase in cell number of spheroid culture with conditioned media from monolayer cultured MSC. BM (black) – 0.7 ± 0.1; AT (grey) – 0.8 ± 0.2; UCM (white) – 1.1 ± 0.2. (n = 3) **(B)** Fold increase of UCM derived spheroids culture in different media. DMEM + 10% FBS (black) – 1.1 ± 0.2; StemPro® MSC SFM XenoFree (white) – 0.8 ± 0.2; DMEM + 5% hPL (grey) – 1.3 ± 0.1; Corning® StemGro® (pattern) – 0.6 ± 0.1 **(C)** Diameter at day 1 and day 6 of culture from UCM-derived spheroids in different media.

spheroids cultured using Corning® StemGro® presented noticeable difference, where a considerable higher number of single/dead cells were observed in culture suspension. This is indirectly supported by the higher standard deviation associated with the diameter calculated after 6 days of culture, 79.4 ± 14.7, showing more heterogeneous spheroid culture due to cell loss. This hints to the possibility that either this medium is not particularly enriched with adhesion proteins that facilitate the cohesion of the spheroids or that the medium does not support cell survival in this 3D configuration. In fact, there is a discrepancy observed regarding the fold increase when comparing with other media, where Corning® StemGro® presents 0.6 ± 0.1, which indicates a cell loss during culture time.

II.2.3.3. Plating density

Since a 3D configuration supposedly mimics more precisely the 3D microenvironment *in vivo*, cell density could be hindering cell proliferation. Actually, the cells in the spheroid conformation virtually "see" a 100% confluence in their proximity, which has been proven to inhibit cell proliferation (Abo-Aziza and A.A, 2017). Considering that the spheroid spatial cohesion cannot be loosened, the remaining option for diminishing the high confluence in proximity would be reducing the plating density of spheroids. To the extreme, the approach used was successive dilution of spheroids generated until single culture was achieved in a 96-well plate.

Nonetheless, analysing 50 single-cell spheroid cultures, the fold increase values obtained was 0.7 ± 0.1 and 0.8 ± 0.1 for AT and UCM derived spheroids, respectively, suggesting that cell proliferation did not occur.

II.2.4. Ki-67 assay

Considering that no effective cell proliferation occurs with the spheroids, it was anticipated that cells within the spheroids might be in a quiescent state, and thus intracellular staining of nuclear protein Ki-6 was performed. This protein is strictly associated with cell proliferation as it can be detected in the cell's nucleus in all active phases of the cell cycle and absent in quiescent cells (Bullwinkel et al., 2006). Both flow cytometry and immunohistochemistry was performed for both 100 cells/sph and 500 cells/sph spheroids (Figure 14). Only for the spheroids initiated with 500 cells/sph Ki-67^{+} cells were detected, with 2.7%, meaning that only a very small percentage of cells are in fact proliferating. These results

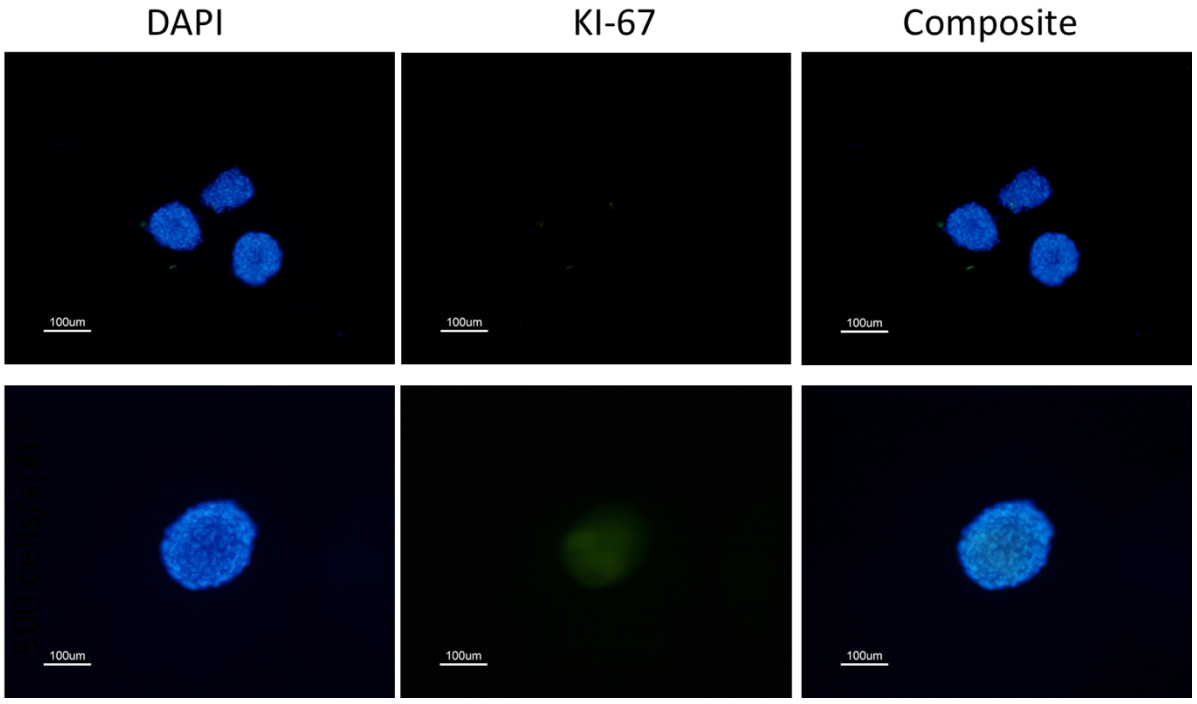

Figure 14 – Immunohistochemical staining with DAPI and Ki-67 at day 6 of culture. Top images are from 100 cells/sph and bottom images from 500 cells/sph. Flow cytometry analysis showed that only for 500 cells/sph the Ki-67 staining was positive (2.7%)

support the higher fold increased obtained for 500 cell/sph when compared to the other spheroid densities tested. However, this level of proliferative cells is not significant to affirm that cells are in fact expanding, proving that the spheroid culture format does not support MSC expansion for the experimental conditions tested herein.

Chapter III

Establishing dynamic culture conditions for the expansion of Adipose-derived Stem/Stromal Cells

Chapter III - Establishing dynamic culture conditions for the expansion of Adipose-derived Stem/Stromal Cells

III. Introduction

The most common support system for adherent cells in dynamic cultures is microcarriers. With size that varies from 50 to 400 microns, these external supports provide large surface area for anchorage-dependent cells to adhere and proliferate. With densities that resemble the water (≈ 1 g/ml), these particles are able to be maintained in suspension resorting to low agitation (Panchalingam et al., 2015). The selection of a microcarrier is a critical step towards the establishment of a successful scalable culture platform. However, the variety of microcarriers, including the new generation of microcarriers, is vast, with differences not only in size but also in the material composition and stiffness, functionalization, porosity and coating (Sart et al., 2013). Overall, there are several options to choose from, and adapt in conformity with the cell type we want to work with. Moreover, the coating provided to the microcarrier plays and important role in controlling cell adhesion. Several microcarriers are available precoated or contain functionalized groups on the surface. Indeed, in monolayer cultures, cell adhesion to culture-treated polystyrene may require further coating (e.g. gelatin) for some media, and thus the performance of some microcarriers could be efficiency improved using the same strategy. On top of that, microcarrier density and cell-to-bead ratio are also variables that affect initial cell adhesion (Chen et al., 2015).

Cell adhesion was shown to be crucial in the early stages of culture, as it affects cell proliferation, hence the need to assure a compatible microcarrier protocol for MSC expansion. A platform was successfully established for the expansion of BM MSC using StemPro® MSC SFM XenoFree media (Carmelo et al., 2015; Santos et al., 2011). In the same study, the authors compared this platform for AT derived MSC expansion and found that it did not support cell proliferation to the same extent. In fact, despite having a similar growth profile, both the adhesion efficiency (36 ± 5 %) and the maximal cell density reached ($1.9 \pm 0.10 \times 10^5$ cells/ml) were significant lower (Carmelo et al., 2015). More recently, another study established an efficient protocol for UCM MSC expansion, using DMEM supplemented with hPL (Soure et al., 2016). Therefore, there is a need to specify and optimize a platform that could efficiently expand AT MSC to the same extent as other sources.

III.1. Material and methods

III.1.1. Isolation of human adipose tissue mesenchymal stem/stromal cells

AT MSC were isolated according with the protocol detailed in section II.1.1.3. An additional step was performed when isolation of AT MSC was performed using hAB supplemented media. After the enzymatic digestion, the sample was incubated with ACK (Ammonium-Chloride-Potassium) for 10 minutes in order to lyse red blood cells, diminishing the presence of blood contaminants. Subsequently, the highly viscous solution was subjected to a filtration step and centrifuged. Afterwards, cells were counted using the Trypan Blue exclusion method.

III.1.2. Conditions for monolayer expansion

Static monolayer cultures were carried out according to the protocol described in section II.1.3.

III.1.3. Media formulation

AT MSC were cultivated in StemPro® MSC SFM XenoFree, alpha Minimum Essential Medium Eagle (α-MEM) (GIBCO, Grand Island, NY) supplemented with 10% (v/v) Human AB serum (hAB) produced in-house, and low glucose DMEM supplemented with three different human platelet lysates: 5% (v/v) UltraGRO™, 5%(v/v) UltraGRO™-ADVANCED and 5%(v/v) UltraGRO™-PURE. UltraGRO™ formulation contains 2 IU/ml of heparin (Sigma-Aldrich, St. Louis, MO 63103, USA). All media were supplemented with 1% A/A.

III.1.4. Microcarrier screening

Table 1 summarizes the microcarriers used for the screening that was performed in order to assess the relative performance with respect to AT MSC seeding efficiency and expansion potential in xeno-free conditions. For the screening, StemPro® MSC SFM XenoFree was the media used. Microcarrier sterilization was performed by autoclaving the microcarriers at 121ºC according to manufacturer guidelines, with the exception of Synthemax II and MicroHex-2D that were already sterile and ready-to-use.

Table 1 – Summary of microcarriers and their characteristics that were used for screening the seeding efficiency and expansion potential of AT MSC.

Microcarrier	Brand	Material	Specific Area (cm^2/g)	Coating
Cultispher-S	Sigma	Gelatin	7200	Gelatin
Plastic	PALL	Cross-linked polystyrene	360	-
Synthemax II	Corning	Class VI polystyrene	360	Synthemax II substrate
Pronectin-F	Sigma	Polystyrene	360	Fibronectin
MicroHex-2D	Fisher Scientific	Polystyrene hexagons	750	Nunclon delta surface
Collagen	SoloHill Engineering	Polystyrene	360	Collagen
FACT III	SoloHill Engineering	Polystyrene	360	Gelatin + Cationic charged
Plastic Plus	SoloHill Engineering	Cross-linked polystyrene	360	Cationic charged
Hillex II	SoloHill Engineering	Polystyrene	515	Modified
Glass	SoloHill Engineering	Polystyrene	360	Silica glass
Enhanced	SoloHill Engineering	Polystyrene	360	-
StarPlus	SoloHill Engineering	Cross-linked polystyrene	515	-

III.1.5. Microcarrier Coating

Plastic, Enhanced and StarPlus microcarriers were coated with CELLstart™ CTS. Incubation was performed for 1 h at 37°C under intermittent agitation (2 min at 650 rpm and 10 min with no agitation) using a Thermomixer comfort (Eppendorf AG, Hamburg, Germany).

III.1.6. Screening study under static conditions

Ultra-low attachment 12-well plates (Costar® Corning®) were used for microcarrier screening. Cells were harvested from the microcarriers at day 1, determining the seeding efficiency, and at day 4, showing the proliferation potential of microcarriers to support cell growth. For each time point, 2 wells were sacrificed for cell count, and cell harvesting performed with TrypLE Express 1X; another well was used for visualization of cell distribution. Microcarrier-cell suspension samples were taken, washed with PBS, and fixed with 1% paraformaldehyde (PFA; Sigma-Aldrich) for 20 min at room temperature. After washing, microcarrier-cell suspension was incubated in the dark with 4′,6-diamidino-2-phenylindole (DAPI, 1.5 g/ml in PBS) for 5 min at room temperature in order to stain the nucleus of the cells (blue) visible under a fluorescence microscope. Fold increase in cell number was calculated as described in section II.1.6.1. (n = 1)

III.1.7. Screening study in spinner flasks

100 ml volume spinner flasks containing a working volume of 80 ml and equipped with 90° paddles and a magnetic stir bar, were used. After adding 12 mg/ml of precoated selected microcarriers to the spinner flasks, 4×10^6 cells were seeded onto a volume of 40 ml of StemPro® MSC SFM XenoFree. For the first 24 h, agitation was set at 30 rpm. From this point onwards, agitation was increased to 40 rpm. On the third day of culture, 40 ml of fresh culture medium was added to the spinner flasks (final volume 80ml). Exchange of 25% (v/v) of culture medium was then performed every day.

A microcarrier–cell suspension sample of 0.5 ml was taken every day from the homogeneous culture in the spinner flask. Microcarriers were then washed with 2ml PBS and incubated with 1 ml of TrypLE Express 1X at 37°C for 7 min at 650 rpm, using the Thermomixer® comfort. In order to stop the enzymatic activity, 2 ml of culture medium supplemented with 10% FBS was added to the cell/microcarrier suspension. Afterwards, the

sample was filtered using a 100 µm Cell Strainer (BD Biosciences). Cell number and viability were determined using the Trypan Blue exclusion method.

DAPI imaging was also performed as described in the previous section, for visualization of cell distribution on the microcarriers.

III.1.8. Optimization of culture conditions in the spinner flask

100 ml volume spinner flasks were used for further optimization of culture conditions, following the inoculation procedure described in the previous section. Feeding regiments, coatings, media and time points were adjusted accordingly to the desired objective.

III.1.9. Characterization of the expanded cells by immunophenotyping

MSC were analysed by flow cytometry using a panel of mouse anti-human monoclonal antibodies against CD14, CD19, CD31, CD34, CD45, CD54, CD73, CD80, CD90, CD105, HLA-DR (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA or Biolegend, San Diego, CA 92121, USA).

Cells were incubated with each antibody for 20 min in the dark and at room temperature, washed with PBS and then fixed using a solution of 1% PFA. Isotype controls were also prepared. A minimum of 10 000 events were collected for each sample and the data was acquired using FACSCalibur (FACSCalibur, Becton Dickinson, San Jose, CA, USA or Becton Dickinson, Franklin Lakes, NJ 07417, USA). CellQuest™ software (Becton Dickinson) was used for acquisition. Analysis was performed using the FlowJo software (Tree Star, Ashland, OR 97520, USA).

III.2. Results and discussion

III.2.1. 2D screening

The first step taken towards the optimization of AT MSC xeno-free microcarrier-based culture was to select the appropriate microcarrier. MSC have been expanded using multiple microcarriers, using animal-derived products such as Cultispher-S and Cytodex 3, to animal-free microcarriers such as Enhanced and Glass (Carmelo et al., 2015; Frauenschuh et al., 2007; Santos et al., 2011; Soure et al., 2016; Yang et al., 2007). In order to select the microcarrier, a 2D screening was performed, using 12-well ultra-low attachment plates under static conditions. As a preliminary screening test, the objective was to assess the ability of AT MSC to adhere to the microcarriers and determine the expansion potential using the StemPro® MSC SFM XenoFree medium. Since these results were not obtained under dynamic conditions, those cannot be directly compared to a 3D dynamic culture behaviour.

In light of this, 12 different microcarriers with different sizes, materials and coatings were tested, summarized in Table 1. Adhesion and fold increase obtained are shown in Figure 15A. Although the objective is to move away from animal-derived products, both Cultispher-S and Collagen microcarriers were tested in order to have a comparison with second generation microcarriers. Statistical analysis distinguishes two different groups with significant difference in adhesion. Pronectin-F, FACT III, Hillex II and Plastic Plus had significant lower cell adhesions when compared the remaining microcarriers ($p<0.05$). MicroHex-2D showed a particular high adhesion, 78.9 ± 0.2%, with comparable values to Cultispher-S 80.4 ± 3.4%; however, when analysing the cell expansion values (fold increase) at day 4, it performed similar or poorer (2.6 ± 0.1) when compared to Plastic (2.7 ± 0.1), Glass (3.0 ± 0.4), Enhanced (3.7 ± 0.1) and Synthemax II (2.7 ± 0.1), that had adhesions of 67.8 ± 1.1%, 64.4 ± 4.4, 63.5 ± 0.1% and 62.5 ± 3.5% respectively. Furthermore, Hillex II, although presented a statistically significant lower initial cell adhesion (46.3 ± 1.8 %), presented a high fold increase (3.6 ± 0.3) which is an important characteristic for cell expansion.

One hurdle in trying to assess proliferation in static cultures is the heterogeneity of the culture, where not only cell distribution per microcarrier is not homogenous since day 1, with the existence of highly saturated microcarriers and relatively low saturated ones, and the early formation of microcarrier aggregates (Figure 15D and E).

Considering both parameters, adhesion and fold increase in cell number at day 4, and the objective to exclude animal-derived products, 7 microcarriers were selected for further optimization: Hillex II, StarPlus, Plastic, Glass, Synthemax II, Enchanced and MicroHex-2D.

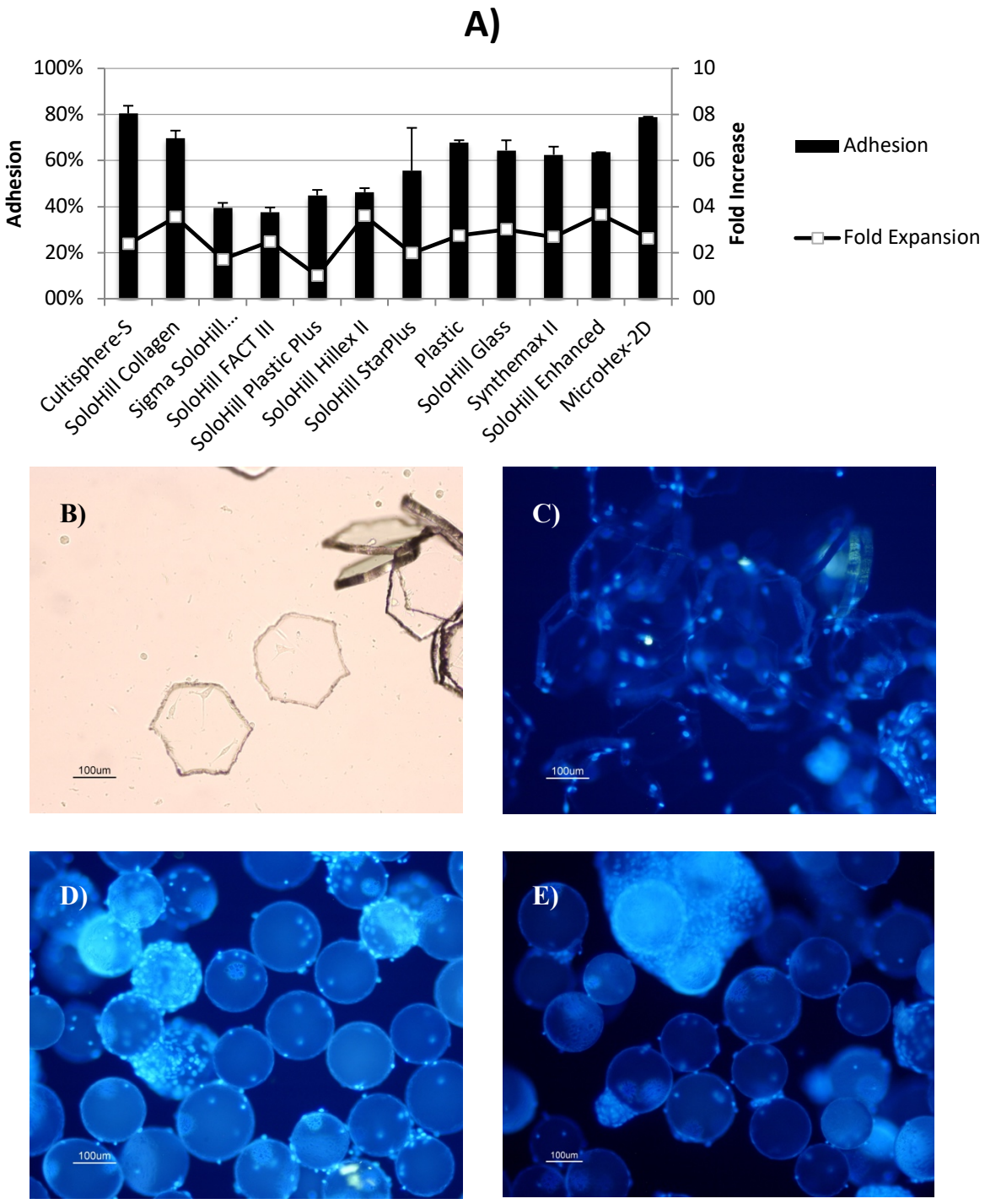

Figure 15 – (A) Cell adhesion and expansion (expressed as fold increase in cell number) obtained for microcarriers in the 2D screening (n = 1); **(B)** Image of MicroHex-2D hexagonal microcarriers under optical microscope; **(C)** DAPI image of MicroHex-2D at day 1; **(D)** DAPI image of StarPlus microcarrier at day 1; **(E)** DAPI image of StarPlus microcarrier at day 4.

III.2.2. Microcarrier screening under dynamic conditions

The seven previously selected microcarriers were then screened in a dynamic 3D culture, using spinner flasks. Growth profiles and cell adhesion for each microcarrier are shown in Figure 16. Statistical analysis shows three different group in regard to adhesion efficiency, one containing Plastic, Glass, Synthemax II and MicroHex-2D, with 36.6 ± 1.7%, 35.6 ± 3.9%, 38.1 ± 4.7% and 50.2 ± 10.9% respectively, other with Hillex II and Enhanced 19.7 ± 1.1% and 21.0 ± 0.72% respectively, and lastly StarPlus with 6.5 ± 4.8%. Considering the 2D screening, the results obtained under dynamic conditions are within the values expected, with the exception of the Enhanced microcarrier that showed comparable values of adhesion in static culture.

Growth profiles were the determinant factor when selecting which microcarrier to pursuit the following studies. In Figure 16A and B, it is represented the growth profile of cells for all tested microcarriers. Figure 16A shows the expansion of AT MSC with Plastic, Glass and Synthemax II microcarriers, which proved to be the ones that supported higher proliferation rates. No significant difference was observed between these three microcarriers, with plastic reaching a 1.4 (± 0.3) x 10^5 cells/ml, Glass reached 1.4 (± 0.4) x 10^5 cells/ml and Synthemax with 1.3 (± 0.3) x 10^5 cells/ml. The cell expansion profiles for the other microcarriers are shown in Figure 16B, where it can be observed that they did not offer great support for AT MSC expansion. In fact, MicroHex-2D despite having a high adhesion efficiency fails to promote cell expansion. On the other hand, Enhanced beads, despite having a very low adhesion efficiency, show the capacity to expand cells, but are not able to reach the same culture densities as the others (6.6 ± 0.3 x 10^4 cells/ml at day 6).

While Plastic, Glass and Synthemax II reach similar cell densities, another parameter that could have distinguished the different microcarriers would be the time period it took to reach those cell densities. However, all three microcarriers show maximal cell density at day 6 of culture, leaving no discernible way to choose one over another. Hence, the Plastic microcarrier was chosen, which has been routinely used in our lab, for different cell types (Badenes et al., 2017; Carmelo et al., 2015).

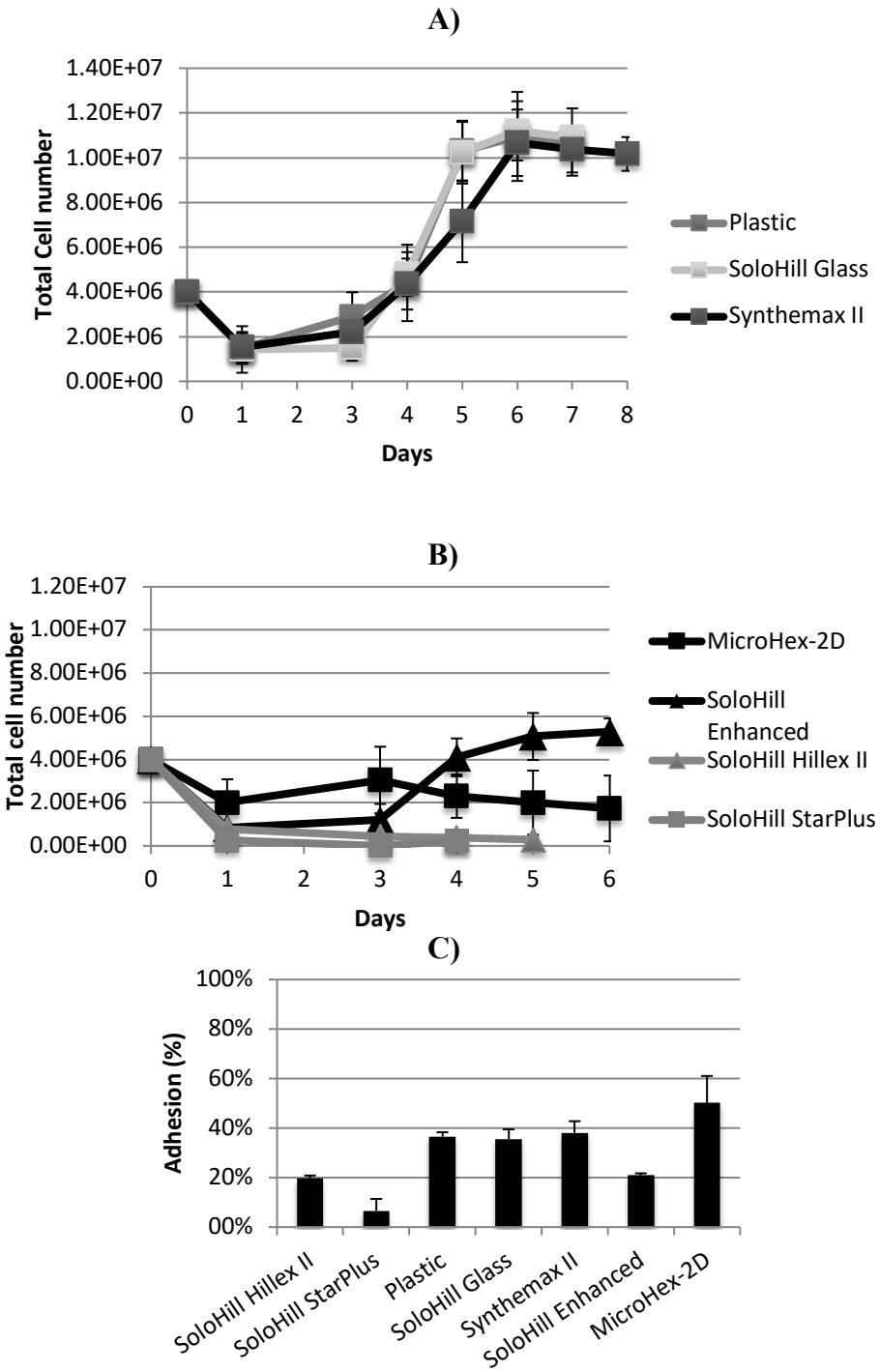

Figure 16 – (A) Cell growth profile under dynamic culture in the spinner flask for Plastic (dark grey), Glass (light grey) and Synthemax II (black) microcarriers, reaching maximal cell density at day 6 with a 1.4 (± 0.3) x 10^5 cells/ml, 1.4 (± 0.4) x 10^5 cells/ml and 1.3 (± 0.3) x 10^5 cells/ml, respectively; **(B)** Cell growth profile under dynamic culture in the spinner flask for MicroHex 2-D (black square), Enhanced (black triangle), Hillex II ((grey triangle) and StarPlus (grey square); **(C)** Initial Cell adhesion (%) of for all microcarriers screened. Hillex II – 19.7 ± 1.1%; StarPlus – 6.5 ± 4.8%; Plastic – 36.6 ± 1.7%; Glass – 35.6 ± 3.9%; Synthemax II – 38.1 ± 4.7%; Enhanced – 21 ± 0.8%; MicroHex-2D 50.2 ± 10.9%. (n = 2)

III.2.3. Optimization of AT MSC expansion using Plastic microcarriers in spinner flask

III.2.3.1. Adhesion in the first 24 hours of culture

Typically, adhesion efficiency is calculated after the first 24h of culture. In fact, some protocols rely on different culture parameters for the first 24h in order to ensure that efficient adhesion is obtained, whether by diminishing/altering agitation, intermittent agitation or holding half volume to increase bead per ml concentration, ensuring more bead to cell contact.

A preliminary study was performed with the objective of determining more precisely the time-point of cellular adhesion to the microcarriers. Plastic microcarriers coated with CELLstart™ CTS were cultured in StemPro® MSC SFM XenoFree. In Figure 17 is shown the adhesion and growth prolife for the first 24 hours of culture. Time points were taken after 6h, 8h, 12h, 16h and 24h. Observing the graph, after only 6h of culture the number of adherent cells counted is approximately the same than after 24h. In fact, all cells not only adhered after 6h but not expansion was observed for the first 24h.

In conclusion, an agitation of 30 rpm for the 24h far exceeds the time needed for cells to attach to the microcarriers, meaning that there is no need to prolong the culture conditions set to improve adhesion beyond 6 hours.

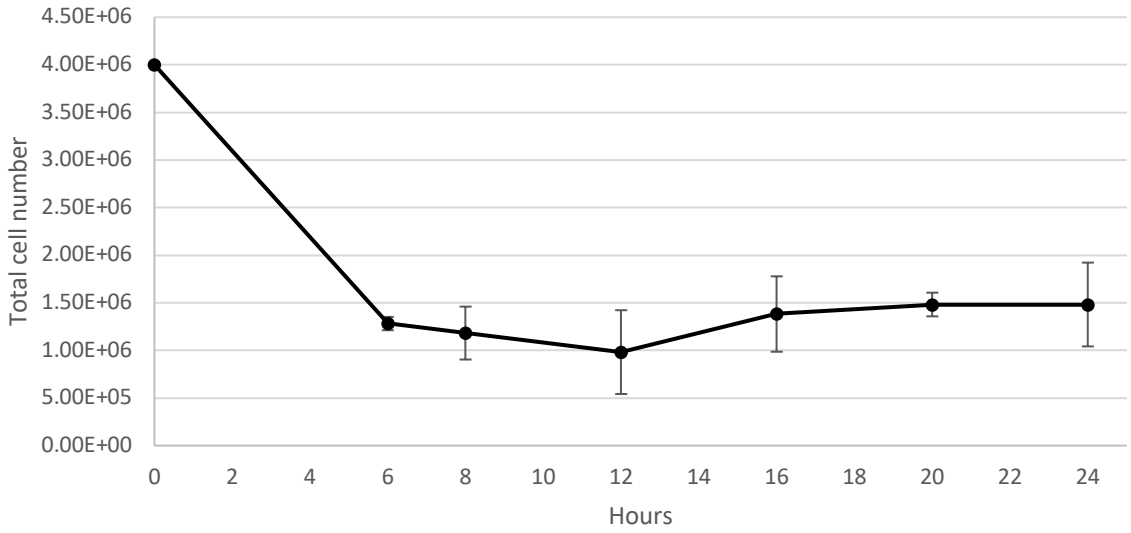

Figure 17 – First 24 hours of culture of AT MSC on Plastic microcarriers coated with CELLstart™ CTS using StemPro® MSC SFM XenoFree medium. Adhesion efficiency was of 32.5 ± 4.9% (mean value of all time points). (n = 1)

III.2.3.2. Comparison between hPL-supplemented medium with StemPro™

The selection of the Plastic microcarrier was performed following the protocol described in the above section, which used StemPro® MSC SFM XenoFree as culture medium and in this case microcarriers were coated with CELLstart™ CTS. However, a protocol using UltraGRO™ developed in our group for the expansion of UCM MSC showed improved results, where both adhesion and expansion (assessed as fold increased) were increased for this source derived MSC (Soure et al., 2016). Similar to the kinetics studies performed in this paper for UCM MSC, isolation and static expansion of AT MSC were studied in our group under the doctoral thesis of the same author (data not shown) (Soure, 2017). When compared to StemPro® MSC SFM XenoFree, it was demonstrated that cell isolation is feasible under xeno-free conditions using hPL-containing medium and cell expansion was improved

The same spinner flask platform used by Soure and colleagues for UCM MSC (Soure et al., 2016) was applied to AT MSC. The difference between both protocols relies in the coating strategy, medium used for expansion and feeding regimen. Regarding the latter, the addition of the the remaining 40 ml (50% of the final working volume) was performed at day 2 instead of day 3, and after day 4 medium exchange was performed every 12 hours instead of every 24h. The coating was performed with DMEM supplemented with 50% UltraGRO™ using the same protocol.

The results with a direct comparison between both protocols are shown in Figure 18. StemPro medium had 32 ± 4% adhesion efficiency versus the 39 ± 3% presented by hPL supplemented medium. Not only there was an increase in adhesion, but also the maximal cell density reached was far superior, 2.68 ± 0.2 cells/ml for UltraGRO™ and 1.96 ± 0.1 cells/ml for StemPro™. Moreover, this cell number was attained at day 5 of culture instead of day 6, reducing the culture time period.

In conclusion, hPL formulation not only promoted a better adhesion but also proved to better promote cell expansion and achieve it in a shorter time period. Nevertheless, this hPL formulation, UltraGRO™ had a major setback: despite being a human-derived formulation, the addition of heparin (from porcine origin) was needed to prevent clotting due to plasmatic coagulation factors, introducing a xenogeneic element in the culture.

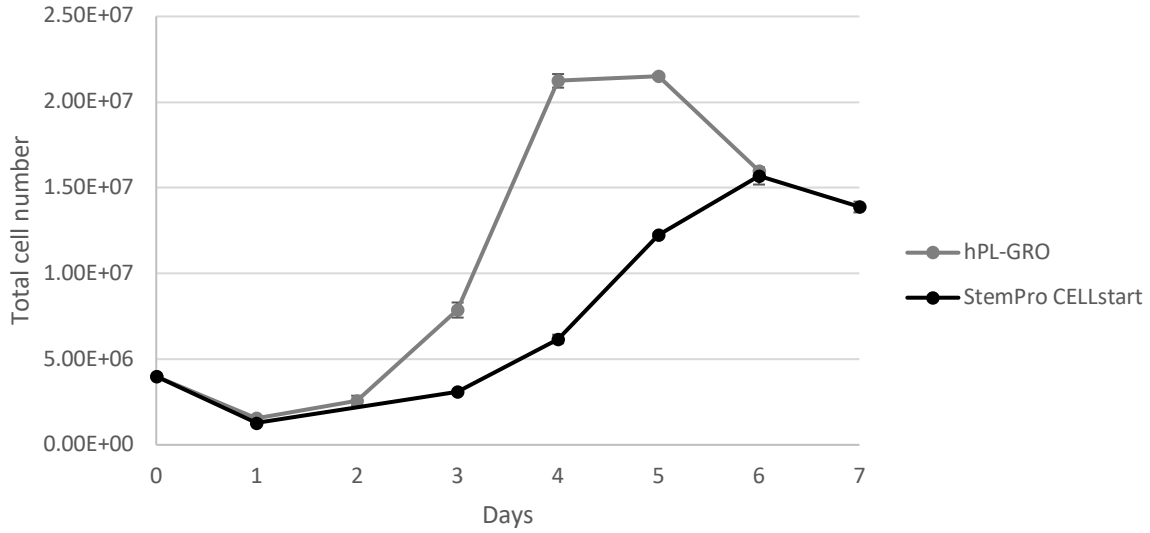

Figure 18 – Growth profiles of AT MSC cultured in StemPro™ with microcarriers coated will CELLstart™ (black) and cultured in UltraGRO™ with microcarriers coated with DMEM + 50% UltraGRO™. StemPro™ - 32 ± 4% with maximal cell density at day 6 of 1.96 ± 0.1 cell/ml; UltraGRO™ - 39 ± 3% with maximal cell density at day 5 of 2.68 ± 0.2 cells/ml (n = 1)

III.2.3.3. Coating strategies

With the previous results using hPL-supplemented medium showing improved adhesion and AT MSC expansion, it was hypothesized that this was due to not only the culture medium being different, but the coating of the microcarriers being more supportive for cell adhesion. Therefore, expansion of AT MSC with StemPro™ culture medium was performed where microcarriers were coated with hPL derived supplement. A different hPL formulation was used, UltraGRO™-ADVANCED, that despite not requiring the addition of heparin, already had been incubated with it during production process, making it non-optimal for clinical usage. For this experiment, Plastic microcarriers were coated with DMEM supplemented with 50% or 5% UltraGRO™-ADVANCED, and then cultures were performed using DMEM with 5% UltraGRO™-ADVANCED (Figure 19A). The improvement on the adhesion efficiency readily noticed, reaching 96% and 92% for 50% coating and 5% coating, respectively. However, despite this improvement in adhesion, maximal cell density was similar to cultures using CELLstart™ coating (1.82 x 10^5 cell/ml for 50% hPL supplemented medium as coating versus 1.78 x 10^5 cells/ml for 5% 50% hPL supplemented medium as coating versus 1.96 x 10^5 cells/ml for CELLstart). However, culture might have been impaired due to high microcarrier aggregation in an early stage of culture (day 3), Figure 19B and C. Microcarrier aggregates are challenging for microcarrier-based type systems, but normally are only

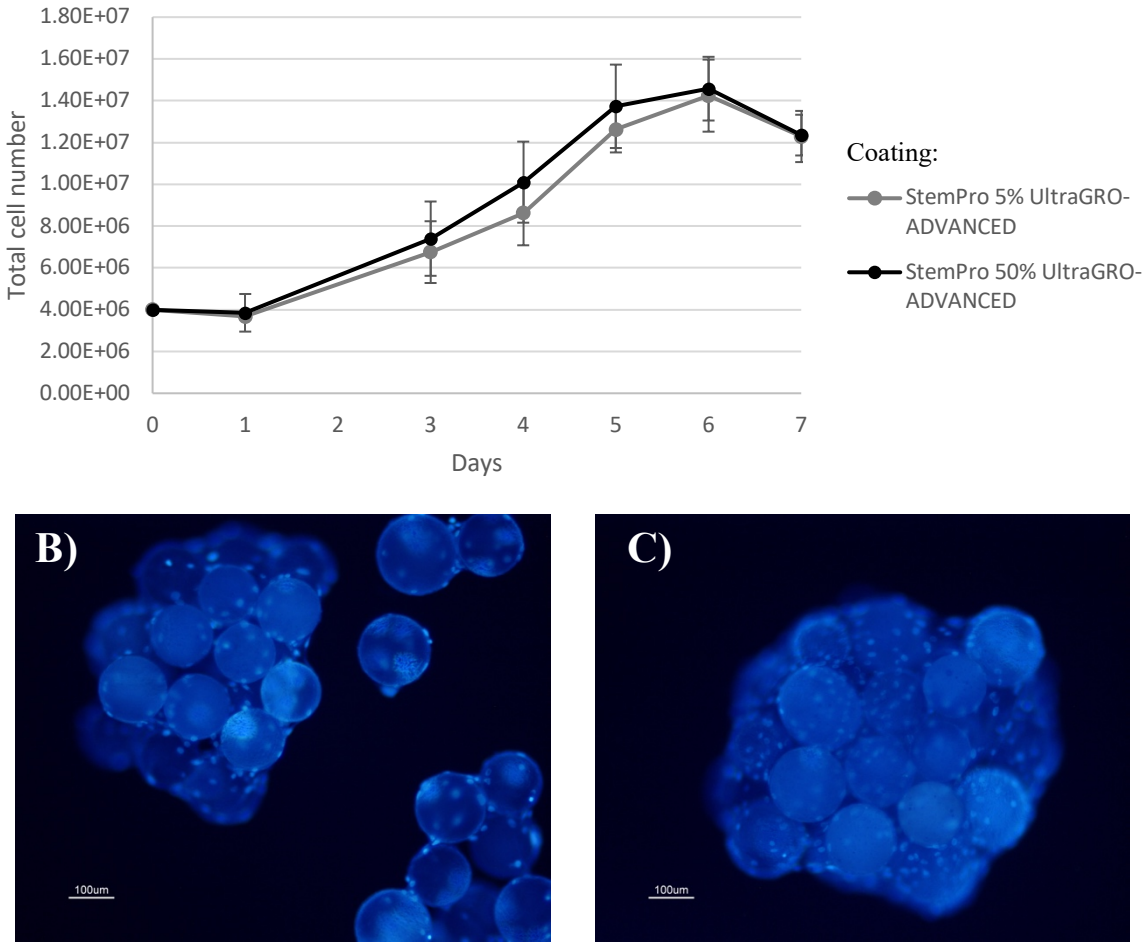

Figure 19 – (A) Growth profiles AT MSC in StemPro™ with microcarriers coated with DMEM supplemented with 50% UltraGRO-™-ADVANCED (black) or 5% UltraGRO-™-ADVANCED (grey); 50% - 96% adhesion and maximal cell density at day 6 of 1.82 x 10^5 cell/ml; 5% - 92% adhesion and maximal cell density at day 6 of 1.78 x 10^5 cells/ml; (n = 1) **(B)** DAPI image at day 3 of 50% coating; **(C)** DAPI image at day 4 of 5% coating.

presented in late stages of culture where cell density per bead is very high. However, using UltraGRO™-ADVANCED, independently of the concentration, a high number of aggregates was present as earlier as day 3 of culture.

Although maximal cell density attained were not different, these results show that the coating of the microcarriers can be performed using a basal medium formulation, DMEM supplemented with 5% UltraGRO™-ADVANCED (compared to a 50% supplementation or the use of hPL as adhesive agent), making it a more cost-effective process.

III.2.3.4. Human AB serum

Within the scope of this thesis, another medium was studied for its capability to expand AT MSC: Alpha Minimum Essential Medium Eagle (α-MEM) supplemented with 10% (v/v) Human AB serum (hAB).

Firstly, three different strategies were tested to coat the plastic microcarriers in order to enhance initial cell adhesion of AT MSC: α-MEM supplemented with 10% or 20% (v/v) of hAB and human fibronectin, Figure 20A. The latter was used as a control due to its wide use as adhesion protein for cell cultivation (Bostancioglu et al., 2017; Kang et al., 2014). Adhesion efficiency ranged from 33% for 10% hAB, 32% for 20% hAB and 37% for fibronectin, resulting in a final maximal cell density of $2.7x10^5$ (day 6), $2.3x10^5$ (day 6) and $2.2x10^5$ cells/ml (day 7), respectively. Although fibronectin showed better adhesion efficiency, it presented a longer lag phase and maximal cell density was lower than either hAB-based coatings. Moreover, hAB-supplemented medium proved to be superior to fibronectin because it reached maximal cell density at day 6 instead of day 7. Considering these results, a 10% hAB coating was selected for subsequent studies.

A direct comparison between hAB supplemented culture medium and StemPro™ was performed, Figure 20B. With 35 ± 2.5% adhesion and maximal cell density 2.6 ± 0.1 x 10^5 cell/ml, hAB medium proved better fitting than StemPro™, with 32 ± 1% and maximal cell density of 1.8 ± 0.1 x 10^5 cell/ml. Interestingly, one donor reached maximal cell density at day 7 as opposed to the others that reached at day 6. During all cultures, cell viability was determined above 90%. Despite the hindrance of not improving initial cellular adhesion, the maximal cell density achieved was higher, indicating that hAB supplemented is a suitable culture medium supplement for AT MSC expansion under dynamic conditions in the spinner flask.

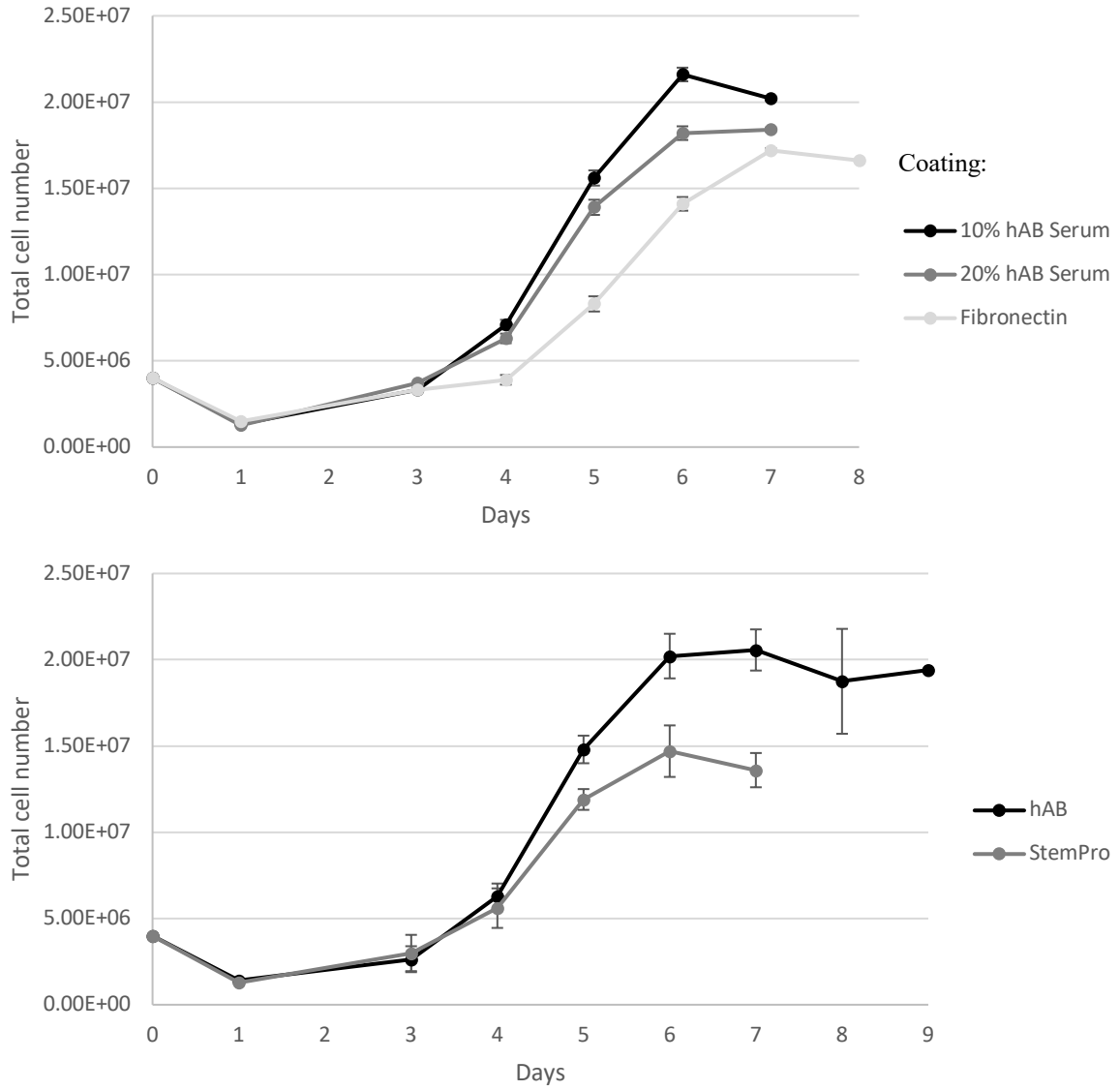

Figure 20 – (A) Growth profiles of AT MSC cultured on microcarriers coated using DMEM with 10% hAB (black) or 20% hAB (dark grey) and fibronectin (light grey) using DMEM 10% hAB as culture medium; 10% hAB - 32% adhesion and maximal cell density at day 6 of 2.7 x 10^5 cell/ml; 20% hAB - 32% adhesion and maximal cell density at day 6 of 2.3 x 10^5 cells/ml; fibronectin – 37% adhesion and maximal cell density at day 7 of 2.2 x10^5 cells/ml (n = 1); **(B)** Growth profile of AT MSC cultured on microcarriers coated using DMEM + 10% hAB (black) using DMEM 10% hAB as culture medium and microcarriers coated with CELLstart (grey) using StemPro; hAB - 35 ± 2.5% and maximal cell density 2.6 ± 0.1 x 10^5 cell/ml at day 7; StemPro - 32 ± 1% and maximal cell density of 1.8 ± 0.1 x 10^5 cell/ml at day 6

III.2.3.5. UltraGRO-PURE supplement

A new formulation of hPL was made available for AT MSC expansion, which was completely xeno-free, UltraGRO™-PURE. A preliminary study was performed using DMEM supplemented with 5% UltraGRO™-PURE as culture media. Coating of the microcarriers was performed using the culture medium itself. Results are shown in Figure 21. The results with this new xeno-free formulation was equivalent to the ones obtained for cultures using the first hPL formulation (UltraGRO™) both in adhesion (42% versus 39 ± 3%), and maximal cell density (2.58 x 10^5 cells/ml versus 2.68 ± 0.2 x 10^5 cells/ml), with the additional advantage of being completely xeno-free.

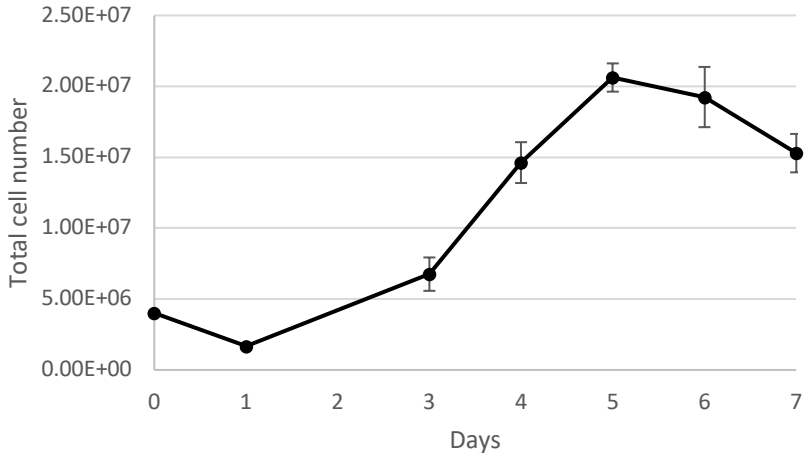

Figure 21 – Growth profile of AT MSC cultured in DMEM supplemented with 5% UltraGRO-PURE with microcarriers coated with culture medium; Adhesion of 42% and maximal cell density of 2.58 x 10^5 cells/ml at day 5.

Our group, by continuing the work developed with hPL UltraGRO™ for UCM MSC expansion, performed optimizations in regard to feeding regiment and initial cell density (data not shown) (Soure et al., 2016). It was showed that by reducing initial cell seeding from 4 x10^6 to 2 x10^6 cells, both adhesion and maximal cell density were improved. Furthermore, with the objective to reduce the number of medium changes due to glucose depletion, a fed-batch strategy with the sole addition of glucose pulses was tested.

For this work, AT MSC expansion was performed with lower initial cell seeding (2 x10^6 cells), and metabolite analysis was performed to verify if a glucose pulse should be taken into consideration, Figure 22A and B. Initial cell adhesion obtained whilst using 2 x10^6 cells

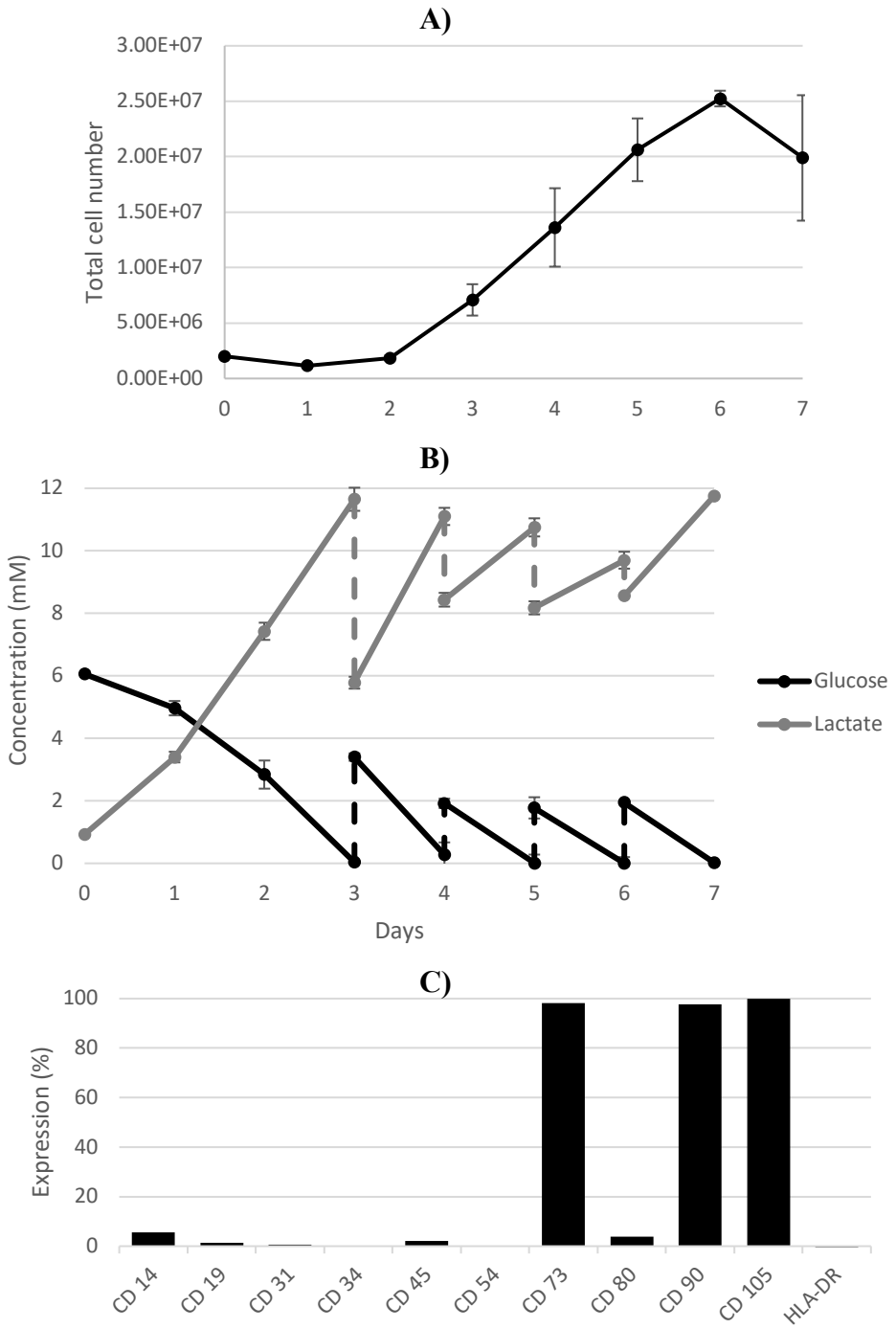

Figure 22 – (A) Growth profile of AT MSC cultured in UltraGRO-PURE where thw inoculum used was of 2 x10^6 cells; Adhesion - 57 ± 7.6%; maximal cell density - 3.16 ± 0.1 x10^6 cells/ml; **(B)** Metabolic profile of AT MSC cultures where glucose (black) and lactate (grey) were analysed; **(C)** Immunophenotyping characterization of the expanded cells

as inoculum was of 57 ± 7.6% and maximal cell density of 3.16 ± 0.1 x10^5 cells/ml. This was a significant improvement compared to previous results, as not only the number of cells needed for inoculation of a scalable system was half, but also higher cell densities were obtained, which are both crucial for clinical translation. Analysis of the concentration of the glucose and lactate

showed the need for improvement of the feeding regimen. In fact, despite lactate never reached inhibitory concentrations (35.4 mM), glucose depletion is observed as soon as day 3. In this regard, a glucose pulse, for a final concentration of 3 g/l, was added alongside with medium exchange every 24h should compensate glucose depletion without the need to increase the medium change rate.

Immunophenotype characterization was performed for expanded AT MSC in spinner flask (Figure 22C) where more than 97% of cells expressed CD73, CD90 and CD105 and less than 2% expressed CD19, CD34, CD45 and HLA-DR. CD14 were expressed in 5.5% of the cells, which is slightly higher than the 2% established by the ISCT (Dominici et al., 2006).

Chapter IV

Ex-vivo Expansion of Adipose-derived Stem/Stromal cells in Stirred tank bioreactors [*]

[1]

[*] Part of this chapter is under preparation for publication as "Successful use of human AB serum to support the expansion of adipose tissue-derived mesenchymal stem/stromal cell in a microcarrier-based platform", by Francisco Moreira, Amanda Mizukami, Lucas Eduardo Botelho de Souza, Joaquim M. S. Cabral, Cláudia L. da Silva, Dimas T. Covas, Kamilla Swiech, (2018)

Chapter IV – Ex-vivo Expansion of Adipose-derived Stem/Stromal cells in Stirred tank bioreactors

IV. Introduction

Scaling-up a culture system in order to obtain cell numbers that can be translated into clinical applications is compulsory. This platform must be time- and cost-effective, and comply to GMP standards (Sart et al., 2010). In the previous chapter, we establish dynamic culture conditions for the expansion of AT MSC in a spinner flask system using plastic microcarriers. However, this system does not feature automation and control, nor it is close to reach clinical-meaningful cell numbers.

Overall, a microcarrier-based expansion under stirred conditions has been reported as easy to scale-up. However, various hurdles need to be addressed in regard culture conditions of this larger system and downstream cell harvesting protocols that can guarantee both cell recovery, as well as cell quality. For instance, the need to provide adequate oxygen supply must be balanced against the detrimental effects of hydrodynamic shear stress generated through stirring. Additionally, the build-up of metabolic by-products and the associated changes in pH need to be taken into consideration (King and Miller, 2007).

Parameters such as oxygen tension, pH and shear stress can influence cell fate (expansion *versus* differentiation), and these are greatly altered when a culture is translated to large scale bioreactor, and thus cell quality must be assured when scale-up is performed.

IV.1. Materials and methods

IV.1.1. Isolation of Adipose Tissue MSC

AT MSC were isolated using either StemPro® MSC SFM XenoFree or α-MEM supplemented with 10% Human AB serum and kept cryopreserved in a liquid/vapour phase nitrogen tank. Adipose tissue samples (n = 3) from liposuction were collected in 500 ml containers and processed in the laboratory. Samples were washed with PBS and homogenized. After resting, two distinct phases were formed, an AT supernatant and an aqueous infranatant phase, being the latter one discarded. This process was performed multiple times until a more yellow and clear solution remained, indicating the absence of erythrocytes and other contaminants. After the washing step, the sample was incubated with 0.1% Collagenase type II at 37 ºC for 30 minutes. The supernatant was discarded, and the pellet was suspended in either one culture medium and centrifuged again. Afterwards, the pellet was resuspended for counting using the Trypan Blue exclusion method.

IV.1.1. Dynamic culture conditions

IV.1.1.1. AT MSC expansion in the Applikon™ mini-bioreactor

A 500-ml mini-bioreactor (Applikon^{TM} Biotechnology) equipped with a three-blade pitched impeller was used. The culture parameters were set to: pH 7.3, 20% of DO by headspace aeration (N_2, O_2 and air) and temperature 37ºC. Pre-coating of plastic microcarriers with α-MEM supplemented with 10% hAB was performed as previously described in section III.1.5. Three bioreactor experiments with independent cell donors were performed (n = 3) for each culture media, StemPro® MSC SFM XenoFree or α-MEM supplemented with 10% Human AB serum. For each run, $12.5x10^6$ cells were inoculated with 20 g/L of pre-coated plastic microcarriers in a final volume of 150 ml. At day 3 of culture, 100 ml of fresh culture medium was added to the bioreactor, and from thereon 25% (v/v) of culture medium was changed every day. Agitation was set at 85 rpm until day 2, 95 rpm until day 5 and 105 rpm afterwards. The increase in agitation was an empirical parameterization to counter-balance the increasing settling rate of the microcarriers due to a gradually higher occupancy by the cells, according to Stokes' law. 2 ml samples of culture were collected daily for cell counting and metabolite analysis.

IV.1.1.2. AT MSC expansion in the New Brunswick® BioFlo

A 1L New Brunswick® BioFlo (Eppendorf®) was used. The culture parameters were set to: pH 7.3, 20% of DO by headspace aeration (N_2, O_2 and air) and temperature 37ºC. Two bioreactor experiments with independent cell donors were performed (n = 2) using DMEM + 5% hPL. Pre-coating of plastic microcarriers was performed with 5% hPL for the first experiment and 50% hPL for the second. For each run, 20 x10^6 cells were inoculated with 20 g/L of pre-coated plastic microcarriers in a final volume of 400 ml. For the first experiment, when cells reached inoculum cell numbers 400 mL of fresh medium was added. Medium was changed when glucose concentration was low (we tried to maintain a 3g/l of glucose). Additionally, when deemed needed, a glucose pulse was added for a final concentration of 3 g/l. Initial agitation was set to 40 rpm but was changed for each run. As described in the previous section, the increase in agitation was an empirical parameterization to counter-balance the increasing settling rate of the microcarriers due to a gradually higher occupancy by the cells. 2 ml samples of culture were collected daily for cell counting and metabolite analysis.

IV.1.2. Metabolite Analysis

Glucose and lactate concentrations were determined in the supernatant samples collected throughout the experiments using an automatic analyser YSI 7100MBS (Yellow Springs Instrument, Yellow Springs, OH).

IV.1.3. Characterization of the expanded cells by immunophenotyping

MSC were analysed by flow cytometry using a panel of mouse anti-human monoclonal antibodies against CD14, CD19, CD31, CD34, CD45, CD54, CD73, CD80, CD90, CD105, HLA-DR (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA or Biolegend, San Diego, CA 92121, USA).

Cells were incubated with each antibody for 20 min in the dark and at room temperature, washed with PBS and then fixed using a solution of 1% PFA. Isotype controls were also prepared. A minimum of 10 000 events were collected for each sample and the data was acquired using FACSCalibur (FACSCalibur, Becton Dickinson, San Jose, CA, USA or

Becton Dickinson, Franklin Lakes, NJ 07417, USA). CellQuestTM software (Becton Dickinson) was used for acquisition. Analysis was performed using the FlowJo software (Tree Star, Ashland, OR 97520, USA).

IV.1.4. Multilineage differentiation potential of the expanded cells

After expansion in the bioreactor, cells were evaluated regarding their potential to differentiate into adipocytes, osteocytes and chondrocytes. MSC multilineage differentiation assays were performed.

IV.1.4.1. Osteogenic differentiation potential

For the osteogenic differentiation assay, cells were plated on a 24-well plate with an initial density of 3,000 cells/cm^2 with expansion culture media until 80-90% confluence is reached. Afterward, StemPro® Osteogenesis differentiation (Brand) culture medium was added. Medium was changed every 4-5 days for a total of 14 days in culture. Osteogenic differentiation was determined by two staining protocols: Alkaline Phosphatase (ALP) and Von Kossa staining.

To perform ALP staining (that allows the evaluation of the activity of early bone progenitors), cells were firstly washed in PBS and then fixed in 2% paraformaldehyde (PFA) for 20 minutes. The cells were then washed with water and incubated with Naphtol AS-MX Phosphate Alkaline Solution 0.25% (Sigma-Aldrich®) with Fast Violet B salt (Sigma-Aldrich®) for 40 minutes, at room temperature. Then, cells were washed four times with distilled water. Cells were observed under an optical microscope.

After ALP staining, cells were incubated with 2.5% silver nitrate solution (Sigma-Aldrich®) for 30 minutes at room temperature for Von Kossa staining. Then, MSCs were washed with distilled water and observed under an optical microscope. Von Kossa staining is used to detect calcium deposits.

IV.1.4.2. Adipogenic Differentiation potential

For adipogenic differentiation assay, cells were cultured in 24-well plate with an initial density of 3,000 cells/cm^2 with expansion culture medium until cells reach 80-90% confluence. Then, medium was changed to StemPro® Adipogenesis differentiation culture medium (brand), being changed every 4-5 days, for a total of 14 days in culture. Afterwards, detection

of adipocytes was performed by Oil Red-O staining. Cells were washed with PBS and fixed with 2% paraformaldehyde (PFA) solution in PBS for 30 minutes at room temperature. Then, MSCs were washed with PBS and incubated with Sudan II-Scarlet for staining of lipid droplets. After washing with distilled water, cells were observed under an optical microscope.

IV.1.4.3. Chondrogenic Differentiation potential

MSCs were plated on 24-well ultra-low attachment plates as small droplets (1-5 μl) at high cell density. The spheroids formed were incubated in a humidified atmosphere at 37°C and 5% CO2 with StemPro® Chondrogenesis differentiation culture medium (Brand), being changed every 4-5 days, for a total of 14 days in culture. Afterwards, cells were analysed for chondrogenic differentiation by Alcian Blue staining. First, cells were washed with PBS and then they were fixed with 2% PFA solution for 30 minutes. Then, cells were washed again with PBS and stained with 1% Alcian Blue (Sigma®) solution for 30 minutes. After staining, cells were washed with distilled water and observed under an optical microscope. Blue staining indicates synthesis of proteoglycans by chondrocytes.

IV.2. Results and discussion

IV.2.1. Applikon mini-bioreactor using hAB supplemented medium

Following the establishment of culture conditions in the spinner flask, we implemented the culture of AT MSC with α-MEM supplemented with 10% hAB in the Applikon mini-bioreactor. With a tighter control over pH, dissolved oxygen, agitation and temperature, we aim to improve even further the expansion of AT MSC under stirred conditions.

Despite using the same coating method and the same initial cell density as in the optimized conditions, the adhesion efficiency in the bioreactor, 22 ± 5%, was lower than when comparing to the spinner flask, 35 ± 2.5%, Figure 23A. However, we were able to achieve the same maximal cell density, also at day 7, of 2.7 ± 0.4 x 10^5 (versus 2.6 ± 0.1 x 10^5 cell/ml in spinner flask), resulting in a higher fold increase (27 ± 8, related to the number of cells that effectively adhered to the microcarriers). Noteworthy, not only all fluid dynamics were different, which can influence adhesion and expansion, but also the initial microcarrier concentration was lower due to a practical impairment that demanded the initial volume not to be 125 mL (50% of total volume) but 150 ml instead. This leads to fewer cell-bead contact that might have hindered cell adhesion. The yield of total cell recovery in the bioreactor at the end of culture was of 43 ± 4%, proving to be a major setback in microcarrier-base systems.

Glucose and glutamine concentrations were measured every day throughout the culture, Figure 23B. As it can be seen, the metabolite profile correlates with the respective growth profile with the stationary phase attained when glucose levels were nearly exhausted at day 8. Although the maximum lactate concentration (3.8 mM) did not reach the inhibitory level for MSC growth (value from ref), the near depletion of glucose on day 7 (0.31 mM) likely impaired cell growth from hereon.

A direct comparison with StemPro® medium was performed, Figure 25. Although initial cell adhesions were similar to the experiments with hAB supplementd medium, 18 ± 3%, this medium proved to be unable to support AT MSC expansion in the bioreactor, for the conditions tested. Three different bioreactors runs failed to expand cells, never reaching the number of cells inoculated to the system. One hypothesis for this phenomenon might be the lack of cytokines or proteins in the serum-free/xeno-free medium commercial formulation that can protect cells from shear stress.

Before and after bioreactor expansion, cells were characterized for the immunophenotypic profile and the capacity to differentiate in different lineages *in vitro*, Figure

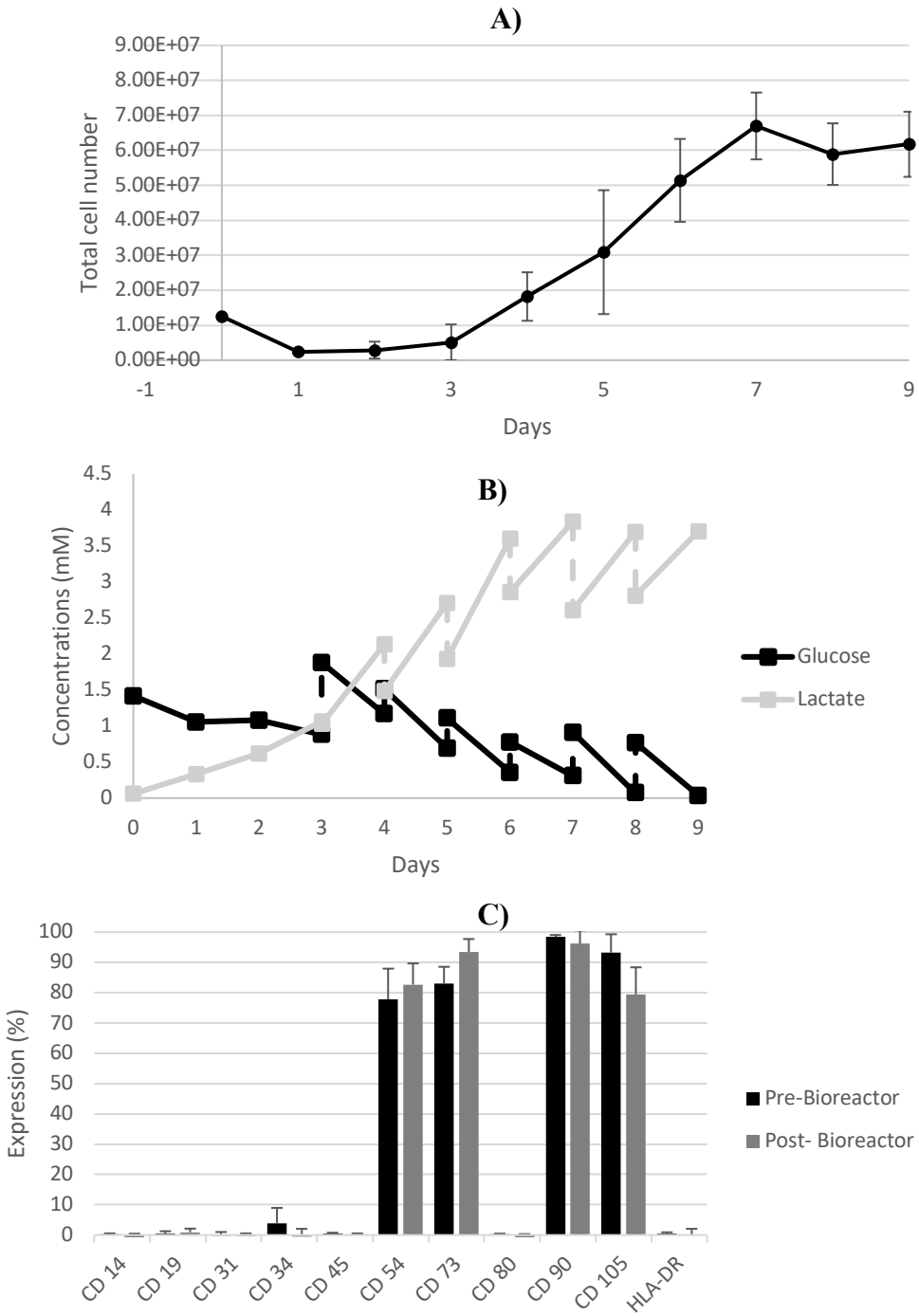

Figure 23 – (A) Growth profile of AT MSC cultured in hAB supplemented medium; Adhesion - 22 ± 5%; maximal cell density - 2.7 ± 0.4 x 10^5 cells/ml; (n = 3) **(B)** Metabolic profile of AT MSC expanded, where glucose (black) and lactate (grey) were analysed; **(C)** Immunophenotyping characterization of pre- and post-bioreactor expansion.

23c. Cell immunophenotypic profile before and after expansion in the bioreactor was not affected. No significant difference was observed in any marker before and after cell expansion. However, the expression of these markers by cells cultured with hAB supplement did not

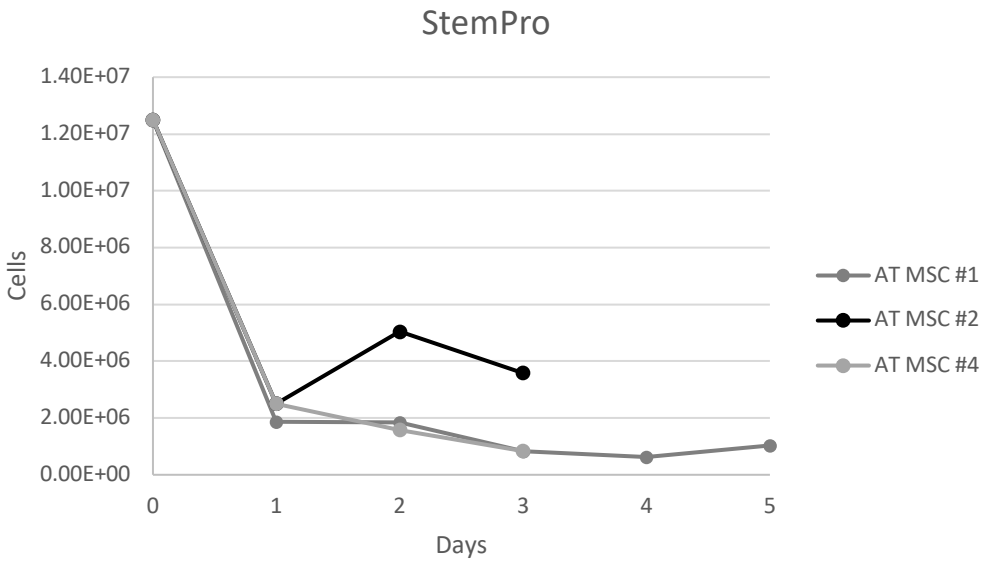

Figure 24 - Growth profile of AT MSC cultured in StemPro; Adhesion - 18 ± 3%. Culture period was followed up to 5 days. (n = 3)

reach the guidelines proposed by ISCT: CD70 was expressed by 83 ± 5% of cells before bioreactor expansion, and 93 ± 4% after; CD105 was expressed by 93 ± 6% of cells before bioreactor expansion, and 79 ± 9% after; CD54 was expressed by 78 ± 10% of cells before bioreactor expansion, and 83 ± 7% after.

Moreover, the expanded AT MSC were shown to maintain the multilineage differentiation potential into the three different lineages, Figure 25A. Differentiation into adipocytes is evidenced by the formation of lipids droplets stained by Sudan II Scarlet; the chondrogenic potential was attested after Alcian Blue staining, hence demonstrating the

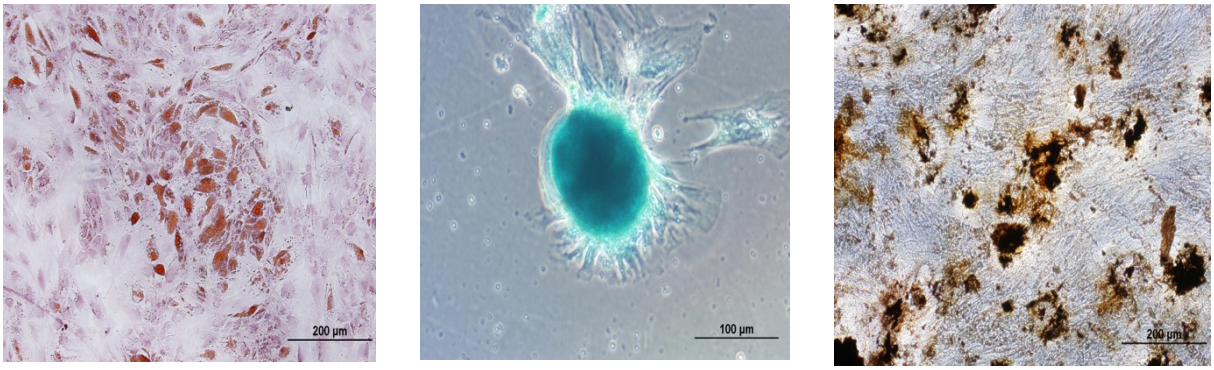

Figure 25 – Multilineage differentiation assays. Adipocyte droplets (left); Chondrogenic potential showing presence of proteoglycans (middle); Osteogenic differentiation showed by accumulation of calcium crystals.

presence of proteoglycans; and the osteogenic differentiation capacity by the accumulation of crystals of calcium oxalate stained with *von Kossa*.

IV.2.2. Bioflo bioreactor using UltraGRO-PURE supplement

New Brunswick® BioFlo bioreactor was used to expand AT MSC with hPL derived media UltraGRO™-PURE. In the first experiment, 5% supplement was used for Plastic microcarrier coating, Figure 26A. However, adhesion efficiency was far lower than expected, 8 ± 1%. The remaining 50% of medium was added at day 4, when cells reached initial seeding cell number, and 25% media changes were performed at day 5, 10, and 14, when glucose concentrations were observed to be lower than 3 mM, Figure 26B. Additionally, a glucose pulse was added at day 11, due to medium change at day 10 not increasing glucose levels significantly, and at day 14 along with media change.

As it can be observed in the growth profile, as culture progress the error in counts increases significantly. The amount and size of microcarrier aggregates affected the culture as sedimentation was inevitable and sample reproducibility was low. This was also confirmed at the end of bioreactor when the whole recovery of cells attached to the microcarriers was performed. Due to the large volume to be enzymatically detached this procedure was performed using TrypLE solution in a spinner flask. Despite de roughness and inaccuracy of this process, the losses were expected to correspond, however, the recovery yield was of 110%, showing the inaccuracy of cell countings the later stages of the bioreactor.

Before and after expansion, AT MSC were characterized for immunophenotypic profile and the capacity to differentiate *in vitro*. Apart from CD105 post-bioreactor that was expressed in only 73 ± 9%, all other positive markers expressed on more than 95% of cells. On the other hand, MSC potential to differentiate into chondrocytes, adipocytes and osteocytes was determined to be present in cell after expansion in bioreactor.

In the second experiment, in attempt to overcome the adhesion hurdle, the coating was performed with 50% hPL supplement (Soure et al., 2016). As shown in Figure 26A, it greatly increased the adhesion, 22 ± 4%, and cells seemed to enter exponential phase. With this coating more aggregation was observed, and in an effort to break and/or prevent formation of more, agitation was increased to 60 rpm. Afterwards, at day 4, a plateau was reached that lasted at least until day 6 where no cell proliferation was observed. No medium changes were done but

a glucose pulse was added in order to prevent glucose concentration to drop. Culture was stopped at day 7, because cells were not expanding.

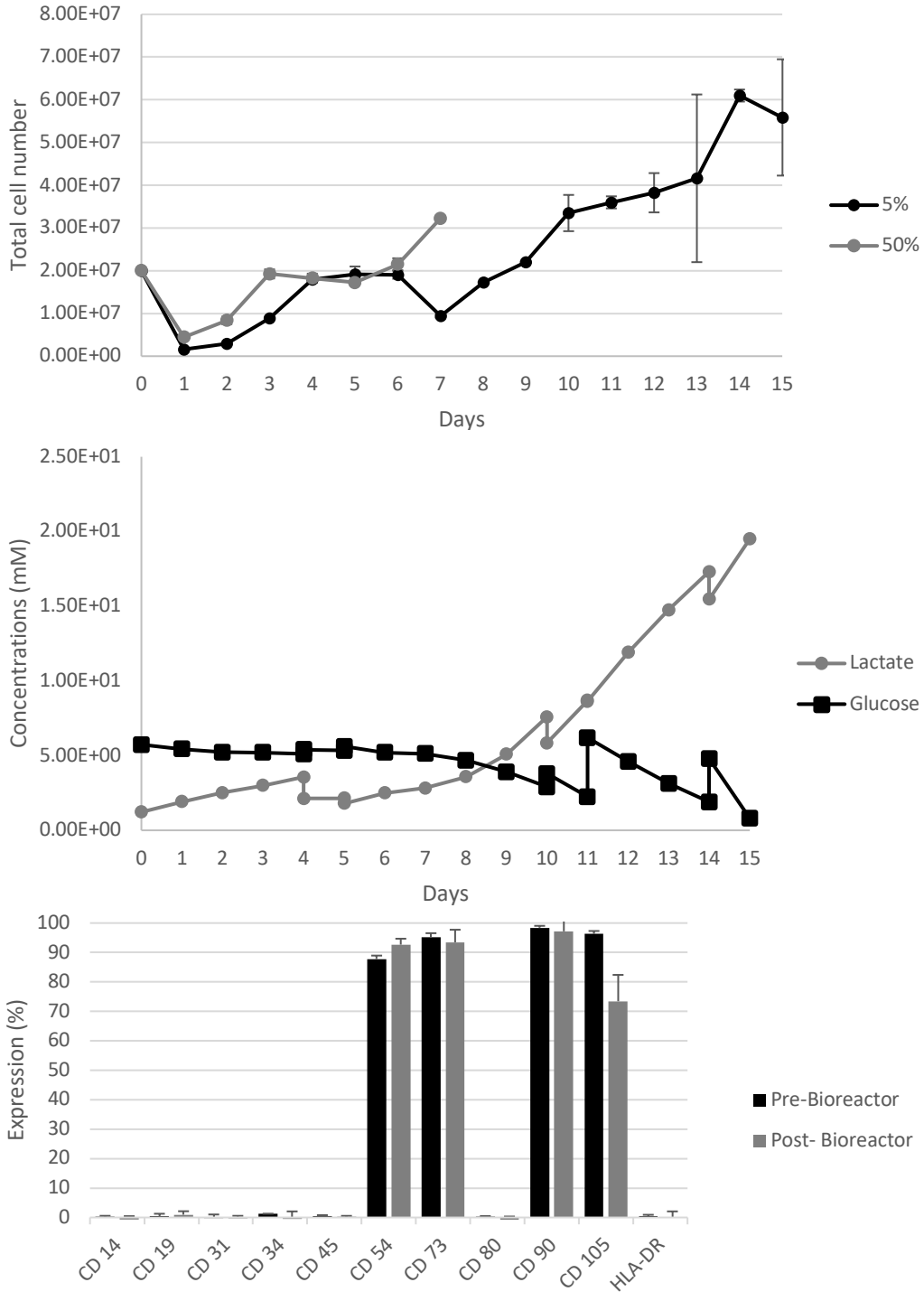

Figure 26 - (A) Growth profile of AT MSC cultured in culture medium supplemented with UltraGRO-PURE, with microcarrier coating of 5% hPL (black) and 50% hPL (grey); 5% - 8 ± 1% adhesion; 50% - 22 ± 4% (n = 1) **(B)** Metabolic profile of AT MSC expanded, where glucose (black) and lactate (grey) were analysed; **(C)** Immunophenotyping characterization of pre- and post-bioreactor expansion.

Chapter V

Conclusion and future trends

Chapter V – Conclusion and future trends

Conclusions and future trends

In chapter II we attempted to expand human MSC cells as 3-D spheroids using several different conditions. Unfortunately, none of the tested conditions proved to be efficient for MSC expansion using the 3D configuration of spheroids. In an attempt to overcome this problem, several methods were applied with little to no success. Different cell sources were used with the thought of using the biological diversity that each MSC source could bring. The size of the spheroids was also studied aiming to eliminate nutrient and oxygen gradients that could lead to a necrotic centre within the spheroid and hence hinder cell proliferation. On top of that, agitation was introduced with the objective of further reducing any heterogeneity or the culture, either to homogenize culture medium and inhibit the by-product accumulation. Different culture media were evaluated for their capability to promote cell proliferation under the spheroid configuration, from animal/serum-derived supplements, to a xeno-free commercially available and human-derived medium. Conditioned medium retrieved from 2D monolayer culture was also utilized with the rationale that secreted growth factors and other proteins could enhance proliferation in the 3D cultivation configuration. These results further showcase that the MSC spheroid potential for clinical applications could not be related to the expansion of these cells but could be related to the priming of these cells for producing higher levels of determined factors. In fact, efforts are being made to further study spheroids with regard to its capability to promote wound healing and angiogenesis, resorting to encapsulation of these spheroids (Costa et al., 2016; Lee and Mooney, 2012). Nonetheless, the usage of a more specific and optimized media could have led to different results (Stella et al., 2014).

Moving into chapter III, we studied the expansion of adipose tissue MSC in dynamic culture. AT is a viable and plentiful source for MSC, which is by-product of other medical interventions that is considered medical waste, being the second most common source of MSC. However, there is a lack in specialized and optimized culture systems for dynamic expansion of AT MSC. This chapter aimed to establish dynamic culture conditions for the expansion of AT MSC in a microcarrier-based platform that was free of xenogeneic agents. For this, the appropriate microcarrier was selected, various coatings and medium were assessed in their capability to support cell expansion and several culture parameters were adjusted.

Two main strategies were established: (i) using culture medium supplemented (5% v/v) with UltraGRO™-PURE, a hPL supplement, combined with microcarriers pre-coated with culture medium, a cell inoculum of $2x10^6$ cells/mL and a feeding strategy featuring a glucose pulse while medium exchange was deemed necessary; (ii) using culture medium supplemented (10% v/v) with hAB serum, combined with microcarriers pre-coated with culture medium, a cell inoculum of $4x10^6$ cells/mL and a feeding strategy of 25% media change every day after day 3 with no glucose pulse. The objective of these optimizations was their translation into a fully controlled bioreactor (Chapter IV).

Further optimization is needed concerning the feeding regimen where a perfusion system would overcome the depletion of nutrients and also reduce the handling of such platforms. On top of that, better characterization and functional assays, such as pro-angiogenic assays, are needed to ensure that expanded AT MSC their innate stem/stromal cells capability.

Lastly, the usage of stirred bioreactors for AT MSC expansion was evaluated in chapter IV. Microcarrier-based cultures present high potential for establishing a scalable and well-defined culture system for AT MSC production under xeno-free conditions. Two different stirred tank bioreactors systems were used for cell expansion: (i) Applikon™ mini-bioreactor and (ii) New Brunswick™ BioFlo.

With the first one, AT MSC were expanded using an in-house made hAB supplement, that showed to support cell expansion. For the second, cells were expanded using UltraGRO™-PURE, where although it shows promise for expansion, still lacks robustness. Adhesion proved to be once again a major setback in these platforms, as less than 25% of inoculated cells were able to adhere to the microcarriers.

The culture medium remains a crucial matter in regards to production of MSC (Mannello and Tonti, 2007). Allogeneic serum, as both media used, tackle the limited availability and high variability regarding large-scale MSC production, and are easier to controlled for quality (Stute et al., 2017). UltraGRO™ has been reported have significant enhanced for expansion kinetics for BM-MSC and AT-MSC when compared to AB serum, which is major advantage for reaching lot sizes in a timelier manner (Kocaoemer et al., 2007). Currently is not known why hPL formulations have a stronger mitogenic effect than hAB or FBS (Coppinger et al., 2004). Furthermore, it has the advantage or being possible to use platelet concentrates after 4-5 days expiration date, reducing cost (Schallmoser et al., 2008). Nonetheless, hAB serum may select for more immature MSC phenotype as reported by Trombi and co-workers (Trombi et al., 2009).

Furthermore, the feeding regimes need optimization, either by tuning the frequency, increasing glucose pulse addition or changing to a perfusion operation. Agitation was a hard parameter to adjust, since we should prevent microcarrier aggregation but at the same time we need to take into consideration the increased shear stress that is being induced over the cells.

When translating to clinical applications, not only the expansion but also the recovery is crucial in obtaining the high number of cells needed. Advancements must be made concerning the downstream process in order to recover cells in a quality-assured manner. For this step we need not only to harvest and separate cells from the microcarriers but also increase concentration of our product (Pattasseril et al., 2013). The development of scalable platforms increases the difficulty of the downstream process as more robust techniques must be applied in order maintain cell viability and function. For cell harvest most studies have been using enzymatic agents (both animal and animal-free reagents) for cell detachment from microcarriers (Caruso et al., 2014; dos Santos et al., 2014; Rafiq et al., 2013). However, enzymatic action has been shown to alter surface markers expression, which could present a problem for viability and/or function, hence the need for careful analysis of these parameters afterwards (Brown et al., 2007). Another option that has been explored would be other type of microcarriers that could themselves be digested without reacting with cells (Das et al., 2018).

Afterwards, a clarification step where microcarriers are removed from the product must be performed. Given the difference in size, microcarriers are usual separated by filtration using size exclusion (Rafiq et al., 2013; Santos et al., 2011). However, there is a need to prevent membrane fouling since large scale platforms present a high number of microcarriers to be removed. With this in mind, tangential flow filtration or close continuous centrifugation have been use with success (Cunha et al., 2013; Hassan et al., 2015; Kolkundkar et al., 2014).

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