Optimization of Mesenchymal Stromal Cell Culture Methodologies Towards the Development of a Cell Therapy for Autoimmune Diseases

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Preface

The work presented in this thesis was performed at the Stem Cell Bioengineering Group, Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period February - December 2020, under the supervision of Dr. Ana Fernandes-Platzgummer and Prof. Cláudia Lobato da Silva. The thesis was co-supervised at Stemlab S.A. (Crioestaminal) by Dr. Francisco dos Santos.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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On a more personal level, thank you to my Biomedical Engineering friends, who best understand the significance of this achievement, for making the last five years much more pleasurable and for being the best support-group one could ask for while writing a thesis. Thank you also to my friends outside the Biomedical Engineering world, who, even though they do not understand completely what it is I have been doing in the last few months, are a constant inspiration.

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Abstract

The optimization of large-scale manufacturing of Mesenchymal Stromal Cells (MSC) towards the development of cell therapy products for the treatment of Graft Versus Host Disease (GVHD) and autoimmune diseases, among others, is an area of growing interest. In this work, cryopreservation using different media was assessed and a post-thawing cell viability of 86.1±1.3% was obtained for 1-month cryopreservation in Cryostor CS5, outperforming the standard culture medium with 10% DMSO (77.4±2.2% for FBS-supplemented medium and 80.3±3.9% for HPL-supplemented medium). Post-thawing immunophenotype characterization revealed no significant alterations. Cells thawed after 2-months cryopreservation showed similar results. Short-term transport solutions, namely fresh culture medium, conditioned culture medium and commercially available media, were also evaluated as a possible alternative to cryopreservation and 2-8 Cellsius was found to be effective in maintaining cell viability above 70% for 5-7 days. Comparatively, both HPL and FBS-supplemented culture medium maintained cell viability above 70% for 3-4 days and the use of conditioned medium was not found particularly beneficial. Alginate encapsulation effectively maintained MSC at RT for 11 days with post-release cell viability of 80.3±1.3%. Additionally, post-encapsulation MSC were able to successfully support hematopoietic stem progenitor cells (HSPC) expansion. Cells retrieved from all transport solutions maintained normal immunophenotype, plastic adherence and multilineage differentiation potential throughout the assay. The Cost of Goods (COG) analysis of the production of an MSC-based product revealed that the number of cryopreservation steps performed and the number of cell passages between them influences the final product's cost/dose. The impact of cryopreservation and transport solutions on MSC quality and functionality, presented in this work will contribute to accelerate clinical translation of these cell products.

Key Words: Human Mesenchymal Stromal Cells, Cell Cryopreservation, Cell Transport, Alginate Encapsulation, Cost of Goods.

Resumo

A otimização da produção em larga escala de células mesenquimais estromais para o desenvolvimento de produtos celulares destinadas ao tratamento de, entre outras, doença do enxerto contra o hospedeiro ou doenças autoimunes, é uma área que tem despertado crescente interesse. Neste trabalho foi investigada a criopreservação destas células em diferentes meios. Um mês após a criopreservação, a viabilidade pós-descongelamento das células criopreservadas em Cryostor CS5 foi de 86,1±1,3%, o que é superior à viabilidade obtida para criopreservação em meio de cultura com 10% DMSO, considerado o standard (77,4±2,2% para meio suplementado com FBS e 80,3±3,9% para meio suplementado com HPL). A caracterização da expressão de marcadores de superfície por parte das células após descongelação revelou a ausência de alterações significativas em relação aos valores normais. Dois meses após a congelação, as células apresentaram características semelhantes. Soluções de transporte a curto-prazo, nomeadamente meio de cultura fresco e condicionado e meios comerciais, foram também avaliadas enquanto alternativa à criopreservação e a solução 2-8 Cellsius revelou-se eficaz na manutenção da viabilidade celular acima dos 70% durante 5-7 dias. Comparativamente, meio de cultura durou 3-4 dias e o uso de meio de cultura condicionado não se revelou vantajoso. Encapsulamento em alginato foi eficaz na manutenção de células à temperatura ambiente durante 11 dias, tendo a viabilidade celular após dissolução do alginato sido 80,3±1,3%. Após o encapsulamento, as células suportaram a expansão de células estaminais hematopoiéticas com sucesso. Todas as soluções de transporte permitiram que as células conservassem a expressão normal de marcadores de superfície, a capacidade de aderir e proliferar em superfícies de plástico e o potencial para se diferenciarem em osteoblastos, adipócitos e condrócitos, durante todo o ensaio. Uma análise dos custos de produção de um produto celular baseado em células mesenquimais estromais revelou que o número de passos de criopreservação e o número de passagens celulares entre eles influencia o custo por dose do produto final. O impacto das soluções de criopreservação e de transporte na qualidade e funcionalidade de células mesenquimais estromais apresentado neste trabalho irá contribuir para a mais rápida aplicação do seu potencial terapêutico na clínica.

Palavras-Chave: Células Humanas Mesenquimais Estromais, Criopreservação Celular, Transporte Celular, Encapsulamento em Alginato, Custos de Produção.

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List of Abbreviations

ARDS	Acute Respiratory Distress Syndrome
AT	Adipose Tissue
ATMP	Advanced Therapy Medicinal Product
ACE2	Angiotensin I Converting Enzyme 2
APC	Antigen Presenting Cells
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BFU-E	Burst-Forming Unit-Erythroid
CFU-GM	Colony-Forming Unit-Granulocyte, Macrophage
CFU-Mix	Multilineage Colony-Forming Unit
CM	Conditioned Medium
CTGF	Connective Tissue Growth Factor
COG	Cost Of Goods
DC	Dendritic Cells
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular Matrix
EMA	European Medicines Agency
EV	Extracellular Vesicles
FBC	Fluidized Bed Centrifugation
FBS	Fetal Bovine Serum
FM	Fresh Medium
FI	Fold Increase
FDA	Food and Drug Administration
GI HPL	Gamma Irradiated Human Platelet Lysate
GMP	Good Manufacturing Practices
GvHD	Graft vs. Host Disease
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
G-CSF	Growth Colony-Stimulating Factor
HCT	Hematopoietic Cell Transplant
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem Progenitor Cells
HGF	Hepatocyte Growth Factor
HSA	Human Serum Albumin
HPL	Human Platelet Lysate
HR	Human Resources
IDO	Indoleamine 2,3-Dioxygenase
IGF	Insulin Growth Factor
IL	Interleukin
M-CSF	Macrophage Colony-Stimulating Factors
MHC	Major Histocompatibility Complex
MCS	Master Cell Stock
MOA	Mechanism Of Action

MSC	Mesenchymal Stromal Cells
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
αMEM	Minimum Essential Medium Eagle alpha
MCP	Monocyte Chemoattractant Protein
MNC	Mononuclear Cells
NK	Natural Killer
NCPD	Number of Cell Population Doublings
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PDL1	Programmed Death Ligand 1
PGE2	Prostaglandin E2
QC	Quality Control
RT	Room Temperature
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SCF	Stem Cell Factor
SLE	Systemic Lupus Erythematosus
SSc	Systemic Sclerosis
TFF	Tangential Flow Filtration
TPO	Thrombopoietin
TGF	Transforming Growth Factor
TMPRSS2	Transmembrane Protease Serine 2
TNF	Tumor Necrosis Factor
UCB	Umbilical Cord Blood
UCM	Umbilical Cord Matrix
VEGF	Vascular Endothelial Growth Factor
WCS	Working Cell Stock
WHO	World Health Organization

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1. Introduction

1.1. Mesenchymal Stromal Cells

Stem cells are characterized by their ability to self-renew and differentiate, being classified according to their differentiation potential[1]. There is an ongoing debate in the scientific community of whether or not the cells first isolated from the bone marrow (BM) by Friedenstein and colleagues[2] can be classified as stem cells. Although their *in vitro* multilineage differentiation capacity lead to the introduction of the term Mesenchymal Stem Cells[3], further studies have shown that this is a heterogenous population with diverse proliferation and differentiation potential[4], leading to the recommendation of the term Mesenchymal Stromal Cells (MSC) by the International Society for Cellular Therapy (ISCT)[5], designation that will be applied henceforth.

MSCs have been the subject of increased research interest due to their particular therapeutic potential and their accessible isolation from multiple sources with few ethical issues associated. A search in the *pubmed.gov* database using the terms "Mesenchymal Stem Cells" and "Mesenchymal Stromal Cells" yielded, in April 2020, 66 050 results, 54 667 of which in the last ten years.

1.1.1. Phenotypic Characteristics

In vitro isolated MSCs are a heterogeneous population of fibroblast-like adherent cells[6] with the ability of self-renewal and possibility of expansion in culture[7]. This heterogeneity, associated with different isolation methods and tissue sources, makes it difficult to clearly define MSCs and to conclude whether different studies using this cell type, and consequently their reported properties, are comparable. This lead the ISCT to propose, in 2006, a set of standards with the goal of clarifying and harmonizing the fundamental characteristics of this special cell population and contributing to the uniformization of research in this field[8]. Thus, MSCs are expected to be adherent to plastic, to express a specific panel of surface antigens (Table 1), measured by flow cytometry in order to identify possible contaminants present after isolation, and to present multipotent differentiation potential into osteoblasts, chondrocytes and adipocytes, under standard *in vitro* differentiation conditions.

Table 1 - Expression of surface markers required to confirm the identity of MSCs.

Positive (≥95% +)	**Negative (≤2% +)**
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR

These constitute only minimal identifying criteria and, as the body of knowledge regarding MSCs grows, more studies are indicating a plasticity of the suggested surface markers[9] and are proposing other possible antigens. Furthermore, the increasing research using different tissue sources indicates that cell properties vary according to the tissue from which they are obtained and suggest the existence of sub-populations with specific properties[10]. For these reasons, it is clear that there is an ongoing need for characterization of these cells and that the current definition should be revisited to incorporate the advances made in recent years.

1.1.2. Sources

Although they were first isolated from the BM[2], MSCs have since been identified in several adult and neonatal tissues, having been isolated, among others, from adipose tissue[11], dental pulp[12], synovial fluid[13], umbilical cord matrix[14], umbilical cord blood[15], amniotic fluid[16] and placenta[17]. As was previously mentioned, although MSCs isolated from different tissues generally present a set of common phenotypical characteristics[18], there are slight variations. In particular, when it comes to the expression of specific surface markers[19], proliferation and differentiation potential[7], these differences may be due to the function of MSCs in the tissue of origin, their microenvironment or whether they originally reside in adult or neonatal tissues[19]. These subtle differences may influence the performance of the cells, depending on the desired application. Thus, it is important to choose the tissue source carefully, taking into account functional factors such as the abundance of MSCs and their expansion potential[18] but also logistical aspects like the invasiveness of the cell procurement process[7].

In the context of clinical application, most pre-clinical research has been carried out in BM-isolated MSCs[20]. For this reason, the BM is considered the gold standard to which alternative approaches should be compared[21]. Nevertheless, MSCs constitute a small percentage of the total number of cells of the bone marrow and this number tends to decrease with increasing age[22]. Furthermore, harvesting of these cells is an invasive and painful procedure[18]. Due to these limitations, there is ongoing research that aims to identify and optimize the use of alternative sources. In the case of clinical application, not all sources are interesting due to poor accessibility[20]. Adipose tissue (AT) constitutes a suitable substitute to BM as it has a higher cell yield[18], is more accessible and resulting MSCs present comparable characteristics, such as similar immunomodulatory properties[19]. Umbilical cord matrix, previously considered to be clinical waste[23], also constitutes a viable alternative as it can be obtained in a non-invasive and painless manner and shows appropriate differentiation and proliferation potential as well as comparable immunomodulatory activity[23] to the other two sources.

Table 2 presents some advantages and disadvantages of using BM, AT and UCM as tissue sources for MSCs isolation. Although, in general, all sources yield MSCs that abide by the minimum criteria defined

by the ISCT[24], it is clear that they possess significant differences that must be considered when selecting the appropriate one for a specific clinical application[22].

Table 2 - Advantages and disadvantages of different MSCs sources.

Source	Advantages	Disadvantages	Ref
Bone Marrow	• Widely characterized; abundance of preclinical data • Isolation methods well established	• Painful and invasive harvest • Low cell yield • Low proliferation rate • Abundance, differentiation potential and maximum life span decrease with age	7,18,20
Adipose Tissue	• High availability and accessible • High frequency of MSCs • Cell yield larger than BM • Higher proliferation rate than BM-MSC • Stronger immunomodulatory effects than BM-MSC	• Abundance, differentiation potential and maximum life span decrease with age	7,18,20
Umbilical Cord Matrix	• Non-invasive harvest • Cell yield larger than BM and AT • High proliferative potential • Unique combination of pre and post-natal MSC properties	• Lack of standard isolation procedure	7,23

1.1.3. Paracrine Action

MSCs are an attractive candidate for cell therapy as they are able to exert a series of beneficial effects, namely tissue repair and immunomodulation, when infused. These effects are known to be mediated by all of the soluble factors actively or passively secreted by MSCs that constitute their secretome[25]. These include growth factors, angiogenic factors, immune regulating factors and extracellular vesicles which contain proteins and microRNA that control target cell functions[26]. The secreted molecules take action not in the secretory cell but in neighboring target cells, which gives MSCs a paracrine action, described by Gnecchi and colleagues in 2006[27].

The secretome of MSCs is of a dynamic nature, not being yet fully characterized. This is due to the fact that it is known to depend on spatial and temporal factors[28] and that many of the molecules are not constitutively produced by MSCs, being secreted in response to specific stimuli, like pro-inflammatory cytokines[29] or hypoxia[30], that MSCs may find in their microenvironment after infusion. As was previously mentioned, the paracrine action of MSCs has different therapeutic effects that span from tissue repair and angiogenesis to immunomodulation and anti-scarring effects[28], modulated by different soluble factors.

1.1.3.1. Immunomodulation

Normally, when an exogeneous agent gains access to the organism, an inflammatory response is activated. The innate immune system constitutes the first line of the host's defense[31] and includes sentinel innate immune cells like tissue resident macrophages and dendritic cells (DCs)[32], which are the main antigen presenting cells (APC) of the immune system[33]. When an inflammatory state is reached, these cells contribute to the production of a series of pro-inflammatory cytokines, chemokines, and other factors that induce a similar state in neighboring cells[32], creating tissue inflammation[31]. These soluble factors attract effector innate immune cells such as neutrophils and natural killer (NK) cells, that kill virally infected, stressed or cancer cells through cytotoxic means[33], to the site of the infection, where they release more pro-inflammatory cytokines[32]. At the same time, adaptive immunity cells are recruited. These include T lymphocytes, which are associated to memory immunity and are antigen specific, and B lymphocytes, which are antigen presenters and antibody producers[33]. These cells only act on a later stage of the immune response as they undergo clonal expansion[31] after antigens are presented to naïve CD4 and CD8 T-cells[32]. An effective inflammatory response is ensured by the synergistic action of innate and adaptative immunity[31].

The immunomodulatory action of MSCs is licensed by inflammation[29]. It is in response to the secreted inflammatory cytokines that MSCs express a series of factors with anti-inflammatory action and, because different states of inflammation yield different responses[29], the immunomodulation of MSCs is considered to be plastic[34].

In general, the immunomodulatory action of MSCs suppresses the activation and proliferation of both innate and adaptive immune cells and inhibits the differentiation of progenitor cells. This is controlled by cell-cell contact and MSC-expressed soluble factors, such as prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), which are some of the main mediators. The anti-inflammatory action of MSCs also contributes to the inhibition of pro-inflammatory cytokines production by the immune system, such as tumor necrosis factor (TNF-α), and the production of anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor (TGF-α)[30,34,35].

Concerning the innate immune system, MSCs are able to block the differentiation of progenitor cells into DCs and their maturation[34], through mediators such as the pro-inflammatory cytokine IL-6 and macrophage colony-stimulating factor (M-CSF)[33]. DCs function is also altered, as MSCs interfere with their antigen presenting abilities[33], which contributes to the inhibition of T-cell activation, as well as the cytokine releasing pattern of these cells[34]: the release of PGE2 by MSCs increases the expression of anti-inflammatory cytokines such as IL-10 and decreases the expression of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and IL-12[36].

The secretion of IDO by MSCs promotes the differentiation of progenitor cells into M2 type macrophages with an anti-inflammatory phenotype, skewing them from M1 type macrophages that present a pro-inflammatory phenotype[7]. These macrophages participate in T-cell suppression[34]. Furthermore, MSC-derived PGE2 activates macrophages and upregulates the secretion of the anti-inflammatory cytokine IL-10[36].

As for NK cells, MSCs halt proliferation and downregulate the activation of IL-2 and IL-5 driven NK-cells[29]. Moreover, cytokine production of NK-cells is changed[35] and MSCs suppress the cytotoxic potential of these effector cells[28].

MSCs also exert effects in cells from the adaptive immune system: MSCs take part in the inhibition of proliferation, differentiation and immunoglobulin secretion of B-cells[36]. Furthermore, besides promoting an indirect inhibition of T-cells through their action on other immune cells, MSCs are also responsible for the inhibition of T-cells activation and differentiation through a direct paracrine action. T Lymphocytes produce pro-inflammatory cytokines like interferon gamma (IFN-γ), when an inflammatory state is established. These soluble factors activate MSCs that increase the expression of anti-inflammatory factors like PGE2, IDO and IL-10[7]. IDO catalyzes the degradation of tryptophan into kynuramine and this factor inhibits the proliferation of T-cells[36] and reduces the total number of cells undergoing activation[19]. MSCs are also able to stimulate the generation of T-cells with regulatory properties (T-regs) from naïve lymphocytes[34] and enhancing their expression of IL-10[19].

1.1.3.2. Trophic Activity

The pro-survival or nurturing factors secreted by MSCs are extremely diverse[36] and their effects range from reduction of tissue damage, for example, in ischemic scenarios[37], and angiogenesis, to support and promotion of tissue repair through the stimulation of neighboring progenitor cells[28], contributing to a general improvement of tissue function in injury sites.

In hypoxic conditions, like the ones found in the first stages of an ischemic lesion, the lack of oxygenation causes cell death, which contributes to an exacerbation of the trauma. These conditions enhance the expression of MSC-derived anti-apoptotic mediators like vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) or insulin growth factor (IGF-I) that act on the adjacent cells promoting survival and limiting field of injury[30]. This contributes to an enhancement of tissue function and improves the plasticity of the remaining tissue[36]. Another aspect that is fundamental for tissue recovery and repair is angiogenesis, that is, the formation of a new vascular network, as it is necessary to supply blood and growth factors to lesion sites[25]. Hypoxic conditions also contribute to an increased secretion of angiogenic factors by MSCs like VEGF and HGF, but also IL-6 and monocyte

chemoattractant protein 1 (MCP-1)[30]. Together, these trophic factors contribute to diminishing cell death and promoting vascularization, working towards tissue recovery.

In wound healing, scarring consists of an excessive and accelerated deposition of extra-cellular matrix (ECM), which prevents complete tissue regeneration[38]. This phenomenon is called fibrosis and consists on the replacement of normal tissue by non-functioning and excessive scar tissue[39]. In certain pathological scenarios like, for example, systemic sclerosis, a fibroblast and endothelial cell dysfunction gives rise to multi-organ fibrosis[40]. Fibrosis is inhibited by MSCs[28] that present an anti-scarring effect[30]. MSCs express anti-fibrotic factors, such as HGF, bFGF and connective tissue growth factor (CTGF)[41], and are able to modulate fibrosis through four strategies: ECM remodeling, immunomodulation, oxidative stress inhibition and inhibition of TGF-β mediated differentiation into ECM-secreting myofibroblasts[36,42]. MSC anti-fibrotic capacity has been demonstrated in several animal models[41].

Another useful aspect of the MSC paracrine action is the support of growth and differentiation of local hematopoietic progenitor cells[30], thereby promoting a healthy hematopoiesis. *In vivo*, hematopoietic stem cells (HSCs) can be found in the BM, where the surrounding microenvironment provides them with signals that control their self-renewal or differentiation[43]. As was previously mentioned, MSCs can be found in the BM and they give rise to most stromal cells present in the BM. They constitute an essential HSCs niche component[44] and are known to secrete hematopoietic molecules like IL-6, growth colony-stimulating factor (G-CSF) and granulocyte- macrophage colony-stimulating factor (GM-CSF)[30]. Thus, the capacity of MSCs to support hematopoiesis has been demonstrated not only in animal models but also *in vitro*[44]. In fact, the use of MSCs-derived feeder layer in HSCs *in vitro* expansion protocols is regarded as a satisfactory strategy to mimic the hematopoietic niche that does not require further supplementation of the culture media [45].

1.1.3.3. Extracellular Vesicles

More recently, some of the beneficial paracrine effects of MSCs have started to be associated with their extracellular vesicles (EVs) secretion. EVs are produced in almost all somatic cell types[25] and can be found in several physiological fluids such as urine, blood, amniotic fluid, among others[46]. They are a heterogenous population of vesicles that are derived from the cellular membrane and encapsulate bioactive molecules like nucleic acids (mRNA and microRNA) and proteins (growth factors, cytokines, enzymes, etc.)[33]. The lipidic-bilayer protects the cargo from enzymatic degradation until it is delivered to its target cells[25].

EVs englobe exosomes and microvesicles. Microvesicles, considered to be large EVs, present a diameter between 100 and 1000 nm and have a non-endocytic origin. Exosomes, which are smaller sized EVs, have a diameter between 40 and 100 nm and consist of endosomes that are released from

cells through exocytosis[46]. These EVs have harnessed a particular interest in the field of regenerative medicine as they have been demonstrated to play an important role in cell-to-cell communication[33,46] and present properties similar to those of MSCs (immunomodulation[33], angiogenesis[25], reduction of cell apoptosis in ischemic lesion animal models and promotion of cell proliferation during tissue repair[36]). Therefore, MSC paracrine action may be partly dependent on the release of exosomes carrying paracrine effectors.

MSC-derived exosomes can be isolated from MSCs of several sources by several methods such as ultracentrifugation and stored in order to safeguard their properties[46]. This allows for the possibility of a cell-free therapeutic approach, as an alternative to the infusion of MSCs[33,46]. The advantages of this strategy are that a cell-free therapy would have fewer concerns regarding immunogenicity, embolism formation and tumorigenicity, as well as simpler storage and handling protocols[33]. In fact, cell-free therapies have presented good results, although it is still not clear whether cell-to-cell contact is indispensable for the paracrine action of MSCs[36].

1.1.4. Therapeutic Potential

Due to their ability to differentiate into several types of mesenchymal cells such as bone, cartilage and adipose tissue, the therapeutic potential of MSCs was initially thought to be their migration and homing to lesion sites, where they would differentiate and engraft, replacing damaged cells[4,47]. This notion was not consistent with the experimental observations, where a great majority of infused MSCs were shown to get trapped in the lungs before they can reach injured tissues and are cleared in a short time post infusion[26]. Furthermore, the complex secretome of MSCs, analyzed in the previous section, exerts a series of beneficial effects that do not involve *in vivo* differentiation or long-term survival. Thus, attention has shifted to their paracrine activity. The primary function of MSCs is now thought to be the secretion of these bioactive factors with regenerative and immunomodulatory properties, working as multi-drug dispensers[7,47]. This therapeutic potential associated to their relative ease of isolation and expansion from several different sources makes them attractive candidates for cell therapy for a series of degenerative and immune diseases[26].

For a cell therapy to be established and deemed safe, the immunogenicity of the product must be determined. Initially, MSCs were regarded as immune privileged, as early studies determined a low expression of major histocompatibility complex (MHC) class I molecules and no expression of class II molecules[48]. This indicated that MSCs could be safely administered, without the need for a MHC match between donor and receiver[49], opening up the possibility of an universal off-the-shelf cell-based therapy. The use of allogeneic cells from an universal donor has clear advantages over the use of autologous cells as they can be made readily available and can originate from young and non-diseased

donors[34]. This is particularly relevant in cases of acute tissue injury, such as myocardial infarction or stroke, for which manufacturing of an autologous cell-based therapy would take too long[50]. In fact, in a scenario where an MSC-based cell product would be commercially available, the mass production and subsequent cryo-banking of allogeneic MSCs is the only feasible strategy to ensure a reasonable cost of goods and sustain a margin derived marketing model[50].

The universal donor hypothesis based on low immunogenicity may, however, be incorrect. More recent data has established that MHC-mismatched MSCs administered to immunocompetent recipients elicit a cellular and humoral alloimmune response that results in rejection, although the implications of this reaction in MSC-based therapy efficacy are not known[49,51]. In fact, the expression of MHC class II molecules, although absent in culture, is likely activated in inflammation sites after infusion[51]. The alloimmune response may explain the rapid clearance of MSCs from the organism post-infusion. This means that their therapeutic action may work through a "hit and run" mechanism[41,51], secreting beneficial factors prior to adaptative immunity rejection[50]. Further research is necessary to better understand the immune response against allogeneic MSCs and how this affects their therapeutic properties, recognizing immunogenicity as a characteristic of MSCs and taking it into account in therapy design[49,51].

MSCs have a considerable therapeutic potential and are being explored as therapeutic strategies for a myriad of different pathologies, from neurological conditions like spinal cord injury, multiple sclerosis, stroke or amyotrophic lateral sclerosis to pathologies related with osteoarthritis or cardiac lesion, among others[52]. In the following sections, the therapeutic potential of MSCs in four specific conditions will be further analyzed: graft versus host disease (GvHD), systemic sclerosis/scleroderma, lupus and COVID-19.

1.1.4.1. Graft versus Host Disease

Graft versus host disease (GvHD) was one of the first therapeutic targets for MSCs-based cell therapy to be explored. Hematopoietic cell transplants (HCT) have been used for more than 50 years to treat blood related malignancies like leukemia, as the graft is effective in eliminating these hematological pathologies[53]. GvHD occurs as a consequence of HCT, when donor T-cells react to HLA proteins on the host cells[53], thus attacking not only the malignancy but also the host's healthy tissues[54], remaining the major complication that arises from this procedure with high morbidity and mortality rates and few therapeutic options, which limits the use of HCT[55]. GvHD can be acute or chronic, depending on how soon the symptoms appear, and acute GvHD presents four grades: I (mild), II (moderate), III (severe) and IV (very severe). Patients with grades III and IV acute GvHD have a poor prognosis[53–55]. Prophylaxis involves immunosuppression and T-cell depletion, although this decreases the graft versus leukemia

effect and increases the risk of relapse[53]. When this fails, the gold standard for treatment of acute GvHD is steroids due to their anti-lymphocyte and anti-inflammatory activity, although not all patients respond to this therapy and less than half achieve complete remission[53].

It is in this context, and following the successful treatment of a child with acute GvHD using allogeneic cryopreserved MSCs by Le Blanc and colleagues in 2004[56], that MSCs, part of the hematopoietic niche and presenting distinct immunomodulation properties, appear as the most widely studied cell product to prevent and/or treat GvHD[55]. The therapeutic rationale is that GvHD, consisting in an immune-pathological disorder, benefits from MSCs' ability to suppress proliferation of alloreactive T-cells[56]. From the large number of phase II and III clinical trials that have been performed in different countries, the majority confirmed safety of MSCs but efficacy outcomes present great variability[54,55], which may be explained by the small number of randomized controlled studies and the lack of standardization of donor selection, MSC generation and dosage[57]. Nevertheless, the potential of BM-MSCs as a second-line treatment for steroid-refractory acute GvHD led to the conditional marketing approval, in 2012, of the first commercial cellular therapy, Prochymal, developed by Osiris Therapeutics (see more information in Table 4Table 4) for pediatric patients, first in Canada and then in New Zealand[54].

1.1.4.2. Autoimmune Diseases

Auto-immune diseases are another common therapeutic target for MSC-based therapies due to their aforementioned well-established immunomodulatory properties. These disorders take place when the immune system loses self-tolerance and acts in an auto-reactive manner. These disorders may be organ specific, like the case of Diabetes type 1, or systemic, like Scleroderma, also called Systemic Sclerosis (SSc), or Systemic Lupus Erythematosus (SLE)[58].

Scleroderma, or Systemic Sclerosis (SSc) is an immune-mediated orphan disease that has a poor medical prognosis[40,59]: high morbidity and mortality and few and ineffective treatment options, these being mainly immunosuppressants, which cause the loss of protective immune response, and symptomatic drugs[58]. There is an unmet clinical need and novel therapeutic approaches are being sought in order to find options with reasonable efficacy, MSCs infusion being one of them. Like other systemic auto-immune disorders, the features of SSc are variable and there isn't yet a definitive understanding of its underlying pathogenic mechanisms[59]. In general, SSc is characterized by three main abnormalities. Firstly, and as was previously mentioned, multi-visceral fibrosis, mainly in the skin and lungs but also in the heart and digestive tract[58], which occurs due to dysregulated repair of connective tissue in response to injury that prompts anomalous fibroblast activation and an excessive deposition of collagen[59]. Secondly, vasculopathy which gives rise to associated comorbidities[41,58], and,

finally, autoimmunity, that is thought to be responsible for the initiation and progression of the disease [59]and is mediated by immune cell activation and production of antibodies specific for autoantigens[58].

MSCs represent an interesting therapeutic approach as, unlike immunosuppressant drugs, they create a transitory and more specific immunomodulatory action. Furthermore, given their angiogenic and anti-fibrotic properties, they hold the potential to counteract all three identified main features of the disease[58]. In two pre-clinical studies using mouse models, Maria and colleagues obtained encouraging results as MSCs infusion was able to reduce lung and skin fibrosis through modulation of the inflammatory and fibrotic process, even in mismatched MSCs[41,42]. This fact is relevant as it is possible that autologous MSCs transplant is not adequate for these cells may be impaired and/or contribute to disease progression[58].

Due to the heterogeneity of the disease, more research, including randomized clinical trials, is still needed before MSCs can be regarded as second line treatment option to SSc. Adding to the promising results of pre-clinical assays, MSCs-based approaches to other fibrotic conditions have been explored in clinical trials, reinforcing the potential of MSCs in the treatment of SSc[58]. The use of MSCs specifically for SSc has not yet been abundantly explored in clinical trials but a phase I/II study (NCT02213705, more information on Table 3) in France was the first to investigate the effects of allogeneic MSCs in severe refractory SSc[58] and, as of June 2020, five studies matched the search "Scleroderma" or "Systemic Sclerosis" and "Mesenchymal" in *clinicaltrials.gov*.

SLE is another heterogeneous auto-immune disease, characterized by the presence of autoantibodies and consequent increased autoreactivity, with inconstant clinical features, including inflammation in multiple organs, such as the renal, neural, cardiovascular, musculoskeletal and cutaneous systems[60,61]. The variable features of SLE were standardized in a set of classification criteria defined by the American Rheumatism Association[62], with the goal of providing guidance in diagnostics. Despite advances being made in terms of management strategies, SLE is still associated with premature mortality[60] and high degrees of morbidity, in particular due to complications associated with active disease in visceral organs, such as lupus nephritis[63]. Like the case of SSc, the standard therapeutic strategy is long-term immunosuppressants, such as corticosteroids[58], which contribute to increase survival but are also associated with severe side effects, including susceptibility to infection, and high toxicity[63]. Furthermore, some patients do not respond to these therapies, being refractory. For these reasons, there is a clear need for more effective and less toxic therapeutic strategies for SLE patients, in particular to those who are refractory to the other available forms of treatment[60,63].

As was mentioned, immunomodulation mediated by MSCs represents a potential alternative approach for auto-immune disorders and SLE is no exception. Pre-clinical and clinical studies by L. Sun and

colleagues[61,63] showed that transplanted allogeneic MSCs (as it is thought that autologous MSCs from SLE patients may be impaired[63]) are able to modulate the immune system, resulting in improvements in disease activity and serological abnormalities, in animal models and refractory SLE patients. This is achieved through the reestablishment of cytokine homeostasis and the upregulation of Tregs expansion, which suppress autoreactive lymphocytes. The data from these clinical studies shows the potential of MSCs to extend the arsenal of therapeutic options for SLE patients[58] but further research and controlled randomized studies are required to unequivocally establish safety and efficacy. Besides L. Sun and coworkers study (NCT01741857, more information on Table 3), there are, in June 2020, nine other studies registered in *clinicaltrials.gov* matching the search "Systematic Lupus Erythematosus" and "Mesenchymal".

1.1.4.3. COVID-19

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that was identified in December 2019 as the cause of the pneumonia affecting several hospitalized patients in Wuhan, China, is the seventh identified family of coronavirus that infects humans, after SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV)[64]. The disease COVID-19, caused by this novel coronavirus, was declared a pandemic by the World Health Organization (WHO) in March 2020, constituting now a global health emergency with 8 525 042 confirmed cases and 456 973 confirmed deaths worldwide (data from the WHO on June 20, 2020). Being highly contagious, this virus spreads through contact with droplets and respiratory secretions or by direct contact[65]. Once transmission takes place, the angiotensin I converting enzyme 2 receptor (ACE2), a receptor widely expressed in almost all tissues of the body, especially in the alveolar type II cells and capillary endothelium, is recognized by the spike protein of SARS-CoV-2[66]. This step, together with the priming of the spike protein by the cellular transmembrane protease serine 2 (TMPRSS2), allow for the entry of the virus in the host cells and its consequent spread[65,66].

In the most severe cases[67], the viral infection stimulates an exacerbated immune reaction in the lungs, characterized by the release of several pro-inflammatory factors, called cytokine storm[66,68]. This is followed by edema, dysfunction of air exchange and acute respiratory distress syndrome (ARDS)[66], creating a context of immune deregulation associated with respiratory distress[67]. COVID-19 patients with ARDS have also been shown to develop multi-organ failure[69], particularly acute cardiac injury, and secondary infection[66]. The currently applied therapeutic strategies are mainly supportive[67–69], there being no vaccine or treatment available, and the mortality rate remains significant among these more severe cases. There is thus a large unmet medical need for safe and effective treatment for these patients[65,66].

The anti-inflammatory and immunosuppressive properties of MSCs have now been widely discussed. It is clear that, once infused, MSCs stay lodged in the lungs where they release a series of factors[69] that contribute, among other things, to lower the concentration of pro-inflammatory cytokines in injury sites. This makes them a potential therapeutic strategy for patients with ARDS, an acute inflammation and injury to the lung and epithelia[70], that may be applied in the most severe COVID-19 cases to reduce the cytokine storm[66]. Pre-clinical studies using ARDS animal models present encouraging results, as the administration of MSCs ameliorates lung function by increasing the clearance of alveolar edema fluid and reducing inflammation[70]. This data supports the therapeutic potential of MSCs in ARDS, despite the fact that most studies involve respiratory diseases caused by bacteria and virus studies are limited to the influenza virus, there being a lack of a definitive SARS-CoV-2 animal model[69].

A pilot study by Leng and coworkers was the first to test the efficacy of intra-venous administration of MSCs in 7 patients with severe COVID-19 pneumonia, in China, in early 2020[66]. The group demonstrated that the MSCs they used did not express ACE2 receptor, nor TMRPSS2, being thus immune to SARS-CoV-2. They found the cells were able to prevent the cytokine storm, which, they hypothesize, may be key for treatment, and inhibit the overactivation of the immune system, promoting at the same time, endogenous repair of the lung microenvironment. A systematic review of 9 studies using MSCs for lung injury by Qu and colleagues[67] concluded that the safety profile of these cells is consistent with that of other trials involving MSCs. Furthermore, they suggest that MSCs may be able to reduce mortality in severe COVID-19 patients through mitigation of inflammatory and physiological damage and improvement of lung function. The global pandemic has prompted a rise in clinical trials investigating possible therapeutic approaches for COVID-19. A search in *clinicaltrials.gov* in June 2020 with the words “COVID-19” and “Mesenchymal” yielded 45 studies. The urgency of finding a possible cure makes the use of well characterized MSCs products with documented safety profiles an advantage. In light of this, several companies are testing their MSCs products that were already on clinical trials for other conditions, to see if they may be useful in the treatment of COVID-19 respiratory inflammation[65]. Examples of this are Mesoblast[71] (view Table 3), with their product Remestemcel-L (Table 4), and Crioestaminal[72], in Portugal.

1.1.5. Clinical trials and approved ATMP

A clinical trial is defined by Friedman and colleagues as a prospective study comparing the effects and value of interventions against a control in human beings[73]. In general, clinical trials are divided into four different phases, although there are studies that merge phases I and II or phases II and III. A phase I study is often referred to as first in man studies as it follows successful pre-clinical tests. Phase I trials involve few participants, often healthy volunteers but also patients who have failed to respond to standard therapies, and their purpose is to evaluate tolerability of the new drug, estimate its maximum

tolerated dose and characterize pharmacodynamics and pharmacokinetics. A phase II trial has a narrower inclusion criterion and enrolls more individuals. The purpose of this phase is to decide whether or not the new intervention should be further developed into phase III. This is done through a preliminary evaluation of efficacy and, again, safety. Phases III and IV precede marketing authorization, enroll a large number of subjects and serve to confirm effectiveness and clinical value of the new interventions, while also assessing possible adverse effects[73].

A search in the clinicaltrials.gov database using the term "Mesenchymal" on 23 June 2020 yielded 1344 clinical studies, mostly phases I and II but also III and IV. A review of the MSCs clinical trials between 2004 and 2018 by Kabat and colleagues[52] reports a great variety of clinical targets, from neurological disorders like spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, stroke and Alzheimer's, to joint problems such as osteoarthritis and rheumatoid arthritis and cardiovascular diseases, thus demonstrating the great therapeutic potential of these cells. The most commonly used sources are BM, AT and UCM. Kabat and colleagues also report that the number of trials registered at *clinicaltrials.gov* increased linearly between 2007 and 2012, after which the increase rate slowed down and actually diminished in 2018. This decrease in the interest for MSCs may be explained by the underwhelming efficacy outcomes obtained in human trials when compared to the positive and encouraging results of the animal model pre-clinical studies. This fact, together with the heterogeneity of protocols employed for MSCs production and administration, as well as different study designs, which makes it difficult to compare between different studies, have hampered the translation of MSCs into the clinic[26,52]. This is also reflected in the number of approved advanced therapy medicinal products (ATMPs), which appears small when compared to the large number of trials. Table 3 shows some illustrative examples of clinical trials registered in the clinicaltrials.gov database for the four previously mentioned disorders: GvHD, SSc, SLE and COVID-19.

Table 3 - Examples of Clinical Trials found in clinicaltrials.gov in June 2020 with the key words "Mesenchymal" and the previously mentioned disorders.

NCT Identifier	Phase	No Enrolled	Target	MSC Source	Industry Sponsored?
NCT02824653	I/II	10	GvHD	Allo, BM	No
NCT02336230	III	55	aGvHD	Remestemcel-L	Yes
NCT01549665	I/II	30	a or cGvHD	Allo, UCM	Yes
NCT01222039	I/II	19	cGvHD	Allo, AT	No
NCT02213705	I/II	20	SSc	Allo, ?	No
NCT04356287	I/II	18	SSc	Allo, UCM	No
NCT01741857	I/II	40	SLE	Allo, UCM	No

NCT04184258	I/II	10	SLE	Allo, Olfactory Mucosa	No
NCT02633163	II	81	SLE	Allo, UCM	No
NCT04366323	I/II	26	COVID-19	Allo, AT	No
NCT04288102	II	100	COVID-19	Allo, UCM	No
NCT04366271	II	106	COVID-19	Allo, UCM	Yes
NCT04371393	III	300	COVID-19	Remestemcel-L	Yes

ATMPs are medicines for human use that are based on genes, tissues or cells and constitute a new opportunity for the treatment of a disease or injury. They can be divided into three types: gene therapy medicines, somatic-cell therapy medicines and tissue engineered medicines[74]. Table 4 presents the ATMPs involving MSCs that have received marketing approvals by the main regulatory agencies in the word.

Table 4 - Approved Advanced Therapy Medicinal Products

Name	**Company**	**Condition**	**MSCs Source**	**Dose**	**Fresh Vs. Cryo**	**Regulatory Status**	**Obs.**	**Ref**
Prochymal (Remestemcel-L)	Osiris Therapeutics	Pediatric aGvHD	BM, Allo	-	-	Conditional marketing approval in Canada and New Zealand in 2012.	No use outside clinical trial context; Osiris Therapeutics' MSCs business was bought by Mesoblast Limited in 2013	[50]
Temcell	JCR Pharmaceuticals	aGvHD	BM, Allo	2 M MSC/Kg	Cryo	Marketing approval in Japan in 2015.	Technology licensed from Osiris Therapeutics	[50,75,76]
Alofisel	TiGenix NV/Takeda Pharmaceutical	Perianal fistulae in Crohn's disease	AT, Allo	120 M MSC local injection ($5*10^6$ MSC/ml)	Cryo with recover step	Marketing approval by EMA in 2018	First MSC therapy approved in Europe	[50,52,76,77]
Stemirac	Nipro Corporation	Spinal Cord Injury	BM, Auto	3.34 M MSC/Kg	Cryo	Conditional approval by PMDA in 2018 in Japan	Time limit of the approval is 7 years	[52,76,78]

Cupistem	Anterogen	Regeneration of joint tissue; indicated for Crohn's fistula	AT, Auto	$3*10^7$ MSC/ml; injection	Cryo	Approved in S. Korea in 2012	-	[76,79]
Neuronata-r	Corestem	ALS	BM, Auto	1 M MSC/ Kg	Fresh	Approved in S. Korea in 2014	-	[76,80]
Cellgram-AMI	FCB Pharmicell	Accute Myocardial Infarction	BM, Auto	5, 7 or $9*10^7$ MSC depending on body weight	Fresh	Approved in S. Korea in 2011	-	[76,81]
Cartistem	Medipost	Knee cartilage defects	UCM, Allo	2.5 M cells/500$\mu\ell$/cm^2	Cryo	Approved in S. Korea in 2012	-	[76,82]
Stempeucel	Stempeutics Research PVT	Critical Limb Ischemia	BM, Allo	?	Cryo	Conditionally approved in India in 2017	-	[76,83]

1.2. Allogeneic MSCs Cell Therapy Development

As was highlighted in the previous sections, the literature indicates that MSCs have a relevant therapeutic potential and can be isolated from donors with relative ease. The development of MSC-based products that can be commercialized and made available for patients suffering from conditions that can be treated with these cells sparks great interest and significant progress has already been made, as can be seen in section 1.1.5.

The production of MSCs for clinical application is not a straightforward process, involving many steps. Two important phases can be defined: the upstream processing, which involves the isolation of MSCs from a tissue source and their large scale expansion with the goal of reaching clinically relevant cell numbers (much greater than the cell yield from most tissue sources[84–86]); and the downstream processing, which involves the recovery of these cells from the expansion platform, their characterization and the "fill and finish" phase, in which the final cell formulation is placed in appropriate storage[87].

The main challenge in the large scale manufacturing of allogeneic cell therapies (previously seen to be more commercially interesting) is the scale up potential of the process[88], as larger cell numbers demand more space and resources to be achieved and the technologies employed in the context of academic research cannot be the same as the ones used to produce cells on a larger industrial scale. Furthermore, the entire biomanufacturing process must comply with the required good manufacturing practices (GMP)[89]. The following sections will review the protocols and technological solutions available for each step of the production of an allogeneic MSC-based product

1.2.1. Isolation methods

Regardless of the selected source, tissue requires processing in order to obtain a homogeneous population of MSCs. The methods employed to do this should be selected carefully as they will influence the characteristics of the population to be expanded *in vitro*[85,90].

BM was the first tissue from which MSCs were isolated, as was previously mentioned. The most common protocol employed to isolate these cells from BM aspirates involves, firstly, a density gradient centrifugation step, using a polymeric solution[18,24,86,91]. This serves to separate the mononuclear cell fraction, which contains a small population of MSCs, among other cells, from the remaining cells present in the BM[86,90]. The MSCs population can then be isolated through different methods, like immune-based cell sorting, less used due to the lack of specific markers and the difficulty in scaling up, and plastic adherence selection, more common[86]. MSCs are anchorage dependent cells and thus will adhere to the culture surface when plated. This more cost-effective method allows for the removal of contaminating cells like hematopoietic cells through consecutive medium changes and

passages[18,24,86,91]. Due to concerns related with sterility and GMP compliance, as well as to establish a certain degree of standardization, there is an interest in the development of fully closed and automated processing systems for BM-MSCs, as well as for MSCs isolated from other sources[86].

MSCs from AT are commonly isolated from lipo-aspirates, previously considered surgical waste. First performed by Zuk and colleagues[92], the most common isolation protocol[85,86,93] involves enzymatic digestion of tissue fragments previously minced and rinsed with phosphate buffered saline (PBS) to remove traces of blood. The adipose tissue is incubated in a solution of collagenase, a proteolytic enzyme, for approximately 30 minutes at 37°C which will degrade the ECM[85]. The enzyme activity is normally stopped by adding culture medium to the solution[94] and, afterwards, a centrifugation step is performed with the goal of isolating the pellet, called stromal vascular fraction, which contains a mixture of cell populations and includes MSCs. The resulting pellet is resuspended and filtered to remove any undigested tissue fragments that may be present[18,93,94]. The isolation of MSCs is done, similarly to BM-MSCs, through plastic adherence, as contaminating cell populations will not adhere to the culture flask and will eventually be washed away in the changes of medium and passages[18,85,86,94].

Despite being widespread, the use of proteolytic enzymes in MSCs isolation may not meet the required standards for clinical application[93] as enzymes affect the quality and quantity of the obtained cells[85,90,95]. Furthermore, different groups use different enzymes, concentrations and digestion times, which does not contribute to standardization[95]. An alternative protocol is the explant method, in which tissue samples are directly plated in plastic culture flasks, where MSCs will eventually grow, allowing for the removal of the tissue some days later[85,90]. This method does not involve the dissociation of the ECM nor excessive mechanical stress to the cells, that remain protected. This is why it is reported to produce a more homogeneous and viable MSC population, higher cell yields and shorter proliferation times, when compared with enzymatic digestion[85,95]. Furthermore, this method is less costly and more easily compliant with GMP[95]. Nevertheless, there is a higher risk of contamination with other cell types and the method is not entirely reproducible, varying with the skill of the operator[90].

The isolation of MSCs from neo-natal tissues, namely from the umbilical cord, is becoming increasingly more common. It is possible to isolate MSCs from several regions of the umbilical cord: amniotic region, umbilical vein sub-endothelium, Wharton's jelly, also called UCM, and umbilical cord blood[85,86], although the most commonly used is UCM. The isolation of MSCs from the UCM starts by removing the blood and the blood vessels and subsequent mechanical dissociation of the tissue. As there is no standardized method for this procedure, different groups resort to different strategies: enzymatic digestion[24,96], explant method[24,97] or a combination of both. Due to the lack of standardization, the applied protocols differ between different groups. In the case of enzymatic digestion, isolations have

been performed comparing the use of different enzymes in different concentrations[98,99] and different digestion times[100].

The culture media employed in the isolation procedures, as well as during expansion, can affect the behavior of the final product[89] and so the formulation should be carefully chosen. Commonly, the culture medium formulations include a basal medium, such as Dulbecco's modified Eagle's medium (DMEM) or Minimum Essential Medium Eagle alpha (αMEM), which contains nutrients like glucose and glutamine, and a supplement containing a cocktail of proteins and growth and adhesion factors essential for the adhesion and expansion of MSCs[86,89]. The gold standard for this supplement used to be 10% fetal bovine serum (FBS) but this posed a series of problems. Firstly, FBS is not chemically defined and presents batch-to-batch variability. Secondly, being a xenogeneic compound, there is a risk of virus and prions contamination as well as the possibility of increasing immunogenicity of MSCs cultured in its presence[84,86,87]. As an alternative to FBS, many groups have explored humanized medium formulations, namely the use of human platelet lysate (HPL), viewed as a safer supplement. HPL is prepared through multiple freeze thaw cycles and sonication from fresh blood or platelet concentrate and holds a mixture of growth factor and adhesion molecules that supports the growth of MSCs[86]. MSCs have been successfully cultured in the presence of this supplement[97] and several studies report the increased proliferative potential of MSCs grown in HPL when compared with FBS[84,86]. In fact, HPL supplementation is able to surpass the disadvantages of FBS and outperform it with regard to MSCs expansion efficiency[85,101]. Nevertheless, HPL is not without disadvantages, remaining concerns regarding human pathogen transmission, ill-definition and batch-to-batch variability[86,87,102]. In the view of GMP compliance for clinical application, it is recommended that an inactivating step targeting viruses is performed prior to commercial release of the HPL supplement. There are several strategies that can be employed to achieve this, one of them being gamma irradiation, which gives rise to gamma irradiated HPL (GI HPL). It has been demonstrated[102] that GI HPL maintains its efficiency and that the inactivation of viruses is effective.

In order to overcome the previously described problems with the most commonly used supplements[85], the goal of medium formulations for MSCs culture is not only a xeno-free solution but also serum-free[87,89]. Although chemically defined formulations containing cocktails of growth factors and bioactive molecules are likely to constitute the future for GMP compliant production of MSCs, more research is still required[86,89].

1.2.2. Expansion methods

After isolation, MSCs need to be maintained in a controlled environment where they will be expanded, that is, propagated without differentiation, in the process that will eventually give rise to the required

number of cells for clinical application. The adequate platform to perform the expansion of MSCs depends mainly on the desired scale. MSCs are typically expanded in a 2D planar culture system, namely culture plates or T-flasks, where cells are maintained as a monolayer, adhering to the plastic surface of the culture vessel and receiving nutrients via contact with the culture medium on one side[86,103]. This is effective only at a small scale as, for larger volumes of cells, the surface to volume ratio of T-flasks and the manual labor they require would quickly make the process inviable.

As can be seen in Table 4, MSCs-based medicines contain, on average, 1 to 2 million cells/kg and some require more than one application. For a large-scale production of MSCs, the establishment of a robust, efficient, cost-effective and most importantly scalable process is still challenging[104]. 2D culture systems such as Cell Stacks work in a similar fashion to the smaller T-Flasks but present larger areas for culture and the possibility of staking multiple layers[103] in an incubator. Using this bioprocessing strategy, it is possible to produce 10 to 100 billion MSCs in a cost effective manner[88] and with a low degree of technical difficulty[101]. Nevertheless, multi-layered flasks are difficult to scale up in order to achieve higher cell numbers as the productivity is limited to the surface area available and higher scales would demand more space in GMP-compliant clean rooms and a certain degree of automation, which would increase the investment[86,105]. Furthermore, the fact that these culture systems do not include online monitoring of culture parameters, are open, and thus susceptible to contamination, and are strongly dependent on manual labor, makes this strategy less than adequate for large-scale clinical-grade commercial production of MSCs[86,88,103,106].

Dynamic culture systems are a more scalable and robust alternative for the large-scale production of MSCs[86,87]. Bioreactors are traditionally employed in the pharmaceutical industry for the production of a variety of therapeutics, with already optimized and cost-effective protocols[105]. These technologies were adapted for the production of cell therapies and there is an abundant number of studies describing positive results regarding the expansion of MSCs in various types of bioreactors, from the simpler and smaller spinner flask[97,107] to larger bioreactors like the packed bed[108], the hallow fiber[109], the vertical wheel[110], the wave motion[111] and the stirred tank[112,113], this last one being the most commonly used. Bioreactors possess several advantages over 2D static cultures, mainly the fact that they are closed systems and allow for the incorporation of sensors to monitor and control key culture parameters like temperature, pH, concentration of oxygen and carbon dioxide, as well as nutrient consumption and metabolite formation. This decreases greatly the need for manual interventions in the culture, which reduces costs and facilitates regulatory approvals[86,105]. Besides these two characteristics present in most bioreactors, the choice of the platform to be used in MSCs expansion should also consider factors like the ease of operation and cell harvest, cell production per growth area and the maintenance of phenotype post culture, the time and cost effectiveness of the technology and

its sterility and/or disposability[103]. In this regard, single-use bioreactors are technologies in which the cell culture is performed in a disposable compartment that is pre-sterilized and certified by the manufacturer[111]. From a regulatory perspective, single-use bioreactors are preferable for clinical applications as they do not require cleaning procedures and eliminate the risk of cross contamination[87]. Furthermore, the certification provided by the manufacturer confers the process a certain degree of standardization and may simplify approvals[111].

Because the currently available bioreactors present very different characteristics and modes of functioning, it is important to optimize certain parameters, such as the concentration of oxygen and the cell seeding density but also the feeding regimen, the substrate used and the speed of agitation, for the chosen platform and the desired application.

The feeding regimen corresponds to the necessity of medium exchange, as the cell metabolism consumes glucose and glutamine and produces lactate and ammonia, two toxic compost that hamper cell development. This means the culture media should be refreshed often with the goal of avoiding the accumulation of these toxic metabolites, but, at the same time, not often enough to dilute to a residual level important autocrine and paracrine signaling molecules produced by MSCs, important for their growth[86], thus establishing a balance. There are four common modes of functioning for bioreactors that can be optimized for each application: batch, in which the nutrients are provided in the initial media and the volume remains constant, repeated batch, which corresponds to a common medium change, fed-batch, in which nutrients are added when depletion is reached and the volume of culture media increases, and perfusion, in which there is a constant flow of medium through the culture, allowing for the supply of nutrients and removal of waste at the same time[114]. The advantage of perfusion-based cultures, like what was established by Dos Santos and colleagues[115] is that there is a reduction of the required culture manipulations[86]. In fact, a larger scale expansion protocol for commercial applications should not include periodical medium changes as it may cause problems regarding GMP compliance and be labor and cost consuming[110]. Fully closed systems like the hallow fiber bioreactor rely on perfusion.

Being adherent cells, MSCs require a substrate to grow. In static bioreactors like the hallow fiber, where cells grow attached to the intracapillary surface of the fibers, or the packed bed, where there is an immobilized scaffold, such as plastic beads, for cells to adhere[88], there is no need for further action. In dynamic 3D cultures, such as stirred tank bioreactors, cell culture is achieved as a suspension of either cell aggregates or microcarriers. Cell aggregates or spheroids mimic the *in vivo* niche of MSCs better than 2D cultures[86,103] and require only dissociation in the downstream processing phase, not needing the separation of two phases like what happens in microcarrier culture. On the other hand,

there are limitations regarding cell proliferation and gases and nutrients diffusion in bigger aggregates[86]. Microcarriers are used more often and correspond to small spherical particles that can be maintained in suspension with the appropriate agitation. In comparison with 2D culture systems, microcarrier-based culture provides a much larger surface to volume ratio[86–88,103] and is simpler to scale up as fresh microcarriers can be added at any time, increasing the available area for cells to adhere. This is also advantageous as it eliminates the need for passaging to increase surface area, which minimizes culture handling as well as the use of enzymatic agents on the cells[86]. There is a wide variety of commercially available microcarriers, with different materials, porosity and surface coatings, which will have different effects on the cells[86–88,103]. Materials range from polystyrene, glass, cellulose and dextran to biodegradable composts like alginate and gelatin, which have the advantage facilitating the downstream processing as the separation step is eliminated. Regarding porosity, there are non-porous, microporous and macroporous microcarriers, which increase even further the available surface area and protect cells from hydrodynamic stresses. As for cell coatings, microcarriers may not receive any or be coated with positively charged functional groups, biologically active proteins like fibronectin and laminin, or collagen and gelatin, in an effort to facilitate cell attachment by emulating the ECM. The disadvantages of microcarrier use are mainly their propensity to aggregate, which is problematic for cell growth and harvest, and the complex downstream processing they demand[88,103]. In that regard, efforts are being made to develop a GMP compliant microcarrier that makes the downstream processing less complex, either by being biodegradable, neutrally charged, or magnetic[87,88].

In the case of suspension cultures, the speed of agitation, corresponding to the speed of the impeller in stirred tank bioreactors, is an important parameter that can influence cells. Once again, a balance must be established, as the agitation rate must be enough to keep the microcarriers in suspension but not too much so that the shear stress felt by the cells is not harmful[86,88]. The consequences of a low agitation speed are the deposit and aggregation of microcarriers, which makes the cell growth and harvest more difficult. On the other hand, too much speed causes turbulent flows inside the bioreactor and elevated shear stress. These phenomena may cause the detachment of cells from the microcarrier as well as changes in cell function[86,88,103]. For these reasons, this parameter must be carefully optimized for each case. The wave motion bioreactor is based on a rocking platform instead of an impeller, which makes the shear stress less significative[111]. Similarly, the vertical wheel bioreactor uses lower agitation rates when compared with stirred tank, which may be translated into potentially lower hydrodynamic stress[110].

Table 5 highlights the characteristics and the advantages and disadvantages of the most commonly used large-scale bioprocessing strategies for MSCs.

Table 5 - Large-scale bioprocessing strategies for MSCs

Platform	Characteristics	Advantages	Disadvantages	Ref
Multi-Layered Flasks	Stacks of static 2D planar culture	• Cost effectiveness • Ease of operation • Simple downstream processing • No shear stress	• Low surface to volume ratio • Difficult to scale up • Labor intensive and time consuming • No inline monitoring of culture parameters • Open system increases the risk of contamination • Non-homogeneous environment causes lot-to-lot variability	86,88,89, 101,103, 104,106
Stirred Tank Bioreactor	3D dynamic culture; cylindrical shaped vessel with an impeller that provides constant movement and where MSCs grow in suspension	• Scalable • Possible to incorporate inline monitoring • Large surface to volume ratio • Less labor intensive than 2D culture • Uniform mixing conditions throughout culture medium	• Microcarrier downstream processing is complex • Shear stress • Not optimized for MSCs production	86,88,103
Vertical Wheel Bioreactor	3D dynamic culture; U-shaped vessel with a vertically rotating wheel that provides agitation to the suspended MSCs	• Lower shear stress when compared with stirred tank bioreactor	• Requires medium change	110
Packed Bed Bioreactor	3D static culture; Immobilized scaffold or bed of carriers in a column through which media is perfused	• Perfusion at low velocities minimizes shear stress • 3D structure mimics more closely the *in vivo* niche • High surface area	• Low harvesting efficiency • Potential for concentration gradients	86,88,108
Hallow Fiber Bioreactor	Stacked semi permeable hallow-fibers; cells grow on the intracapillary surface and medium with nutrients and oxygen flow on the other surface.	• Large surface to volume ratio • Low shear stress • Efficient mass transfer • Online monitoring of culture parameter and full automation	• Costly consumables • Low harvesting density • Not scalable	88,109

		• Closed system does not require clean room		
Wave Bed Bioreactor	3D dynamic culture; rocking platform promotes wave-like motion in the culture media inside a single use cell bag	• Low hydrodynamic stress • Moderately scalable	• Possibility of turbulent flows and microcarrier deposition	[111]

1.2.3. Population Doublings

Because MSCs exist in limited numbers in their tissues of origin and donors are not abundant, an essential step of MSC production for clinical applications is their *in vitro* expansion with the goal of obtaining a significant number of cells for administration[116]. However, the limited *in vitro* proliferative capacity of human cells, described by Hayflick and colleagues[117], is well established and MSCs, being no exception, cannot be propagated indefinitely, with the risk of acquiring a senescent phenotype. Senescence is caused by in vitro aging which occurs regardless of the age of the donor from which cells were isolated. Although the mechanisms behind this phenomenon are not fully understood, a relation between telomere length and proliferative capacity of MSCs has been identified, with studies reporting a loss of up to 2kb in telomere length when MSCs are passaged *in vitro*[118]. Furthermore, the maintenance of the telomere is a key characteristic of immortal cell strains[119].

Senescent cells present morphological, phenotypical and genetic alterations[118]: firstly, they possess an enlarged and flattened appearance; secondly, they display an upsurge in expression of senescence associated genes; and finally, they exhibit a reduced replication capacity, diminished differentiation potential, reduced migration and homing ability and reduced immunomodulatory properties[116,118,120]. Because extensive passaging negatively affects biological and functional properties of MSCs, influencing their quality and effectiveness as therapeutic agents, it is of the upmost importance to monitor cell senescence and to consider the influence of *in vitro* culture and cell aging on the goal of obtaining high-quality MSCs when designing new therapeutic strategies and their manufacturing process[118,120].

Number of cell population doublings (NCPD) is a more informative form to report the age of a culture than the number of passages or the duration of the culture[121]. It can be calculated using Equation 1**Erro! A origem da referência não foi encontrada.**, where N_t corresponds to the number of cells harvested at a time point t and N_i corresponds to the number of cells seeded at the initial time point i[118,121]. Several studies have tried to identify the upper limit of NCPD before cells enter senescence, in order to determine to what extent may MSCs be safely expanded in vitro. Studies by Bruder and colleagues[122] and Bonab and colleagues[123] obtained, from inoculation to senescence, a mean cumulative NCPD of 38±4 and 30 respectively. Other studies report that the maximum NCPD for MSCs is between 40 and 50[119], between 30 and 40[116] or even between 15 and 30[118]. Although there is no

clear information regarding the NCPD used in the approved MSCs-based products (Table 4)[116], knowing that therapeutic properties of MSCs deteriorate with cell aging in vitro, it is recommended that MSCs manufacturing strategies try to minimize the number of passages and, consequently, the NCPD, in order to ensure that the cells used are functionally healthy[116,120].

Equation 1 - Formula to calculate the number of cell population doublings.

$$NCPD = 3{,}322 * \log\frac{N_t}{N_i}$$

1.2.4. Cell Recovery

The expansion process of MSCs lasts until clinically relevant cell numbers are reached, after which a purification process is required in order to recover the cells from the expansion platform. This purification process encompasses several steps[86,124]: firstly, cell clarification, which includes detachment from whatever surface they are adherent to and separation from the microcarriers, when applicable. Secondly, cell concentration, which involves sample volume reduction with the goal of reaching the clinically required cell concentration, and, finally, cell washing, to reduce impurity levels to less than 1ppm[125,126].

Both in 2D and 3D culture systems, the first step following expansion is cell detachment. Because the detachment from microcarriers is more complex than in 2D planar cultures, due to the different cell adhesion patterns and a phenomenon called microcarrier bridging[104], the choice of microcarriers for cell expansion should consider not only the attachment efficiency but also the ease of detachment[86].

Cell detachment is normally performed using proteolytic enzymatic agents such as porcine-derived trypsin which targets the cell surface adhesion proteins, interfering with the cytoskeleton and causing the cells to assume a rounded shape and detach[87,127]. For therapeutic applications and due to the required GMP compliance, TrypLE, a recombinant trypsin derived from microbial fermentation[127], is commonly used as an animal-free alternative to trypsin[86]. Nevertheless, several studies report that prolonged exposure to enzymatic agents may inhibit cell growth, cause cell damage or alter their immunophenotype, which is undesirable as the maintenance of functionality for therapeutic purposes is a priority[86,127–129]. For this reason, is it important to adapt the choice and concentration of the dissociation agent to the type of culture, choice of microcarriers and the application of the final product, so that exposure time may be optimized[87]. Furthermore, alternative methods have been developed in order to try and minimize cell exposure to enzymatic digestion: mechanical agitation may aid dissociation and speed up the process[86,128] and thermosensitive microcarriers[130] represent a gentler strategy as cells can potentially detach just by decreasing the temperature, with no enzymatic component needed[86,127]. Biodegradable microcarriers are another alternative that have spurred some attention[131,132] as, besides facilitating the cell detachment process, it also reduces the complexity of

the downstream processing, eliminating the need for a step to separate the microcarriers from the cell suspension[86,87]. Normally, in this case, detachment occurs with the action of two enzymes, one for cells' surface proteins and the other no dissolve the microcarriers[87]. The enzyme activity must be quenched before moving forward in the downstream process. It is important to consider what to use for this purpose, as the commonly applied spent media may not meet regulatory standars[87,129].

While the cell detachment process can be performed inside the bioreactor where expansion occurred, MSCs-microcarriers separation, as well as the remaining steps of the downstream processing demand specific technologies to be performed, especially in larger production scales. These technologies must preserve cell viability and phenotype and be scalable, closed and automated, ideally integrating several steps of the process[125].

Separation is normally achieved through size exclusion, as microcarriers (125-212µm) are larger in size when compared with MSCs (15-20µm)[86,87,124]. In smaller scales, cell strainers or vacuum filtration using filters composed of nylon or polypropylene, with different pore sizes (higher pore sizes have higher cell recovery yields[124] but these may let microcarrier debris through, which will contaminate the cell products[87]) are effective[86,124]. For larger production scales, technologies such as the Harvestainer, involving a bag of 25L, where microcarriers are retained, inside a 200L bag for MSCs[87,125], are suitable alternatives. Similarly, tangential flow filtration (TFF), well established in the production of biopharmaceuticals[124,125], can be adapted (as in this case cells are the final product instead of a mere by-product) and, with hallow fiber filters with the adequate pore size, used for cell separation[86,125], as well as for the remaining MSCs downstream processing, integrating MSCs clarification, concentration and washing, which is advantageous as reduces loses between steps and decreases the footprint in general[125,133]. Fluidized bed centrifugation (FBC) may also be employed, taking advantage of the different densities of the two phases[125].

The obtained MSCs suspension is not yet suitable for administration or cryopreservation, requiring still a concentration step with the objective of meeting a certain cell concentration. For smaller quantities, a simple centrifugation would suffice[86] but, as cell numbers increase, this would become a bottleneck. As was previously mentioned, for larger scales, this concentration step can be performed using either continuous or discontinuous TFF[86,87,125] which can achieve results of 6 fold volume reduction[133]. It can also be performed resorting to FBC[86,87,125] which, according to Hassan and colleagues, becomes the most cost-effective option in larger scales of production[125]. Cell washing, required to make sure no unwanted particles end up in the final cell product, can be integrated in both of the previously mentioned technologies[87], in particular in TFF when operating in diafiltration mode[125]. Different

techniques such as expanded bed chromatography have also been explored to improve the washing step[126].

1.2.5. Cryopreservation Methods

For the clinical translation of MSCs, especially if an off the shelf product is the goal, cryopreservation is a key step, as it allows for the establishment of banks, where cells can be safely stored in controlled conditions for prolonged periods of time while maintaining their biological properties[134]. This may be useful during the manufacturing process, when master and working cell banks are created, or to store the final product until patient administration.

Following the downstream processing steps previously described, the final product formulation is obtained and placed in appropriate cryopreservation vessels such as screwcap cryovials or cryopreservation bags, to facilitate storage and shipping logistics. In commercial scale MSCs manufacturing, this is done resorting to filling technology to ensure homogeneity and standardization[135].

In cryopreservation, temperature is decreased until cell metabolism is halted[136]. The rate at which temperature is lowered is relevant for post-thaw cell viability and thus should be optimized[134]. During the freezing process, extracellular water turns to ice, which causes the concentration of extracellular solutes. If the cooling happens slowly, cells are able to restore osmotic balance by losing water by exosmosis, which allows them to concentrate intracellular solutes and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. This way, the cell dehydrates and avoids the formation of intracellular ice, which may lead to cell death[137]. On the other hand, if cooling happens too quickly, the cells are not able to lose water fast enough resulting in internal freezing[138]. Because intracellular ice formation must be minimized to prevent cell rupture, typical cryopreservation methods employ a slow cooling rate of 1/2°C per minute[106]. This can be achieved with cooling devices that use either conduction or convection based approaches to ensure a homogeneous and controlled rate freeze, or resorting to a controlled rate freezer, better suited for larger scale production, which can be programmed to perform specific cooling protocols in a consistent and reproducible fashion[134]. Although the slow cooling is the most common method, nontraditional approaches with other freezing rates have been explored, such as a method using an ultra-rapid cooling[136]. Independently of the cooling rate selected, MSCs are stored in liquid nitrogen tanks at -196°C after freezing[106].

The cryopreservation media is another aspect that must be considered, as it also has effects on post-thaw viability. As was mentioned above, the formation of intracellular ice is detrimental for cell viability and should be avoided. To this end, cryoprotective agents are added to the cryopreservation solution as they avoid cell structural disruption during freezing and thawing[138]. There are several possibilities

available for what cryoprotectant to use and these can be divided into two types: intracellular agents, which penetrate inside the cell and prevent the formation of ice, such as dimethyl sulfoxide (DMSO), glycerol and ethylene glycol, and extracellular agents, which reduce the osmotic effect that occurs in the freezing procedure, like sucrose, trehalose, dextrose and polyvinylpyrrolidone[137]. DMSO is the choice for cryoprotective agent in most protocols found in the literature despite the fact that it is cytotoxic at room temperature (RT)[138]. In fact, it is generally accepted that 10% DMSO together with culture media and adequate supplementation is an effective cryopreservation solution and preserves MSCs function[121,138]. Nevertheless, because DMSO present in thawed cells may cause adverse effects in patients who receive MSCs infusion[134] which is problematic for GMP compliance, and because DMSO removal prior to infusion is a complex and costly procedure[134], efforts have been made towards developing cryopreservation solutions that minimize[139] or eliminate[140] the presence of DMSO, resorting to alternative cryoprotectants which achieve lower cell viability yields but are safer for clinical applications[106,137,141]. In order to comply with GMP, the presence of serum should also be carefully considered.

The cryopreservation protocol should be optimized according to final product application with the goal of ensuring that the therapeutic properties of MSCs are maintained post-thawing, so that they can be readily administered.

1.2.6. Quality Control

As was seen in the previous sections, the manufacturing of allogeneic MSCs-based products is a complex process and many factors may impact the phenotype and therapeutic properties of MSCs, particularly in larger production scales[142]. For this reason, it is important to adequately characterize the MSCs being produced in order to ensure that cells from different lots present similar attributes and that they are safe, effective and ready to be released for clinical use[88,89]. The quality of allogeneic MSCs products should thus be controlled in key steps of the manufacturing process, from donor selection to product release, in accordance with GMP and guidelines provided by regulatory agencies[143].

Quality control (QC) testing encompasses identity, viability, sterility, purity, stability and potency[88,143]. The first three, considered the minimum tripartite components of release criteria for MSCs-based products in early phase clinical trials, are well established notions[142]: identity can be easily verified with the ISCT minimal criteria[8], resorting to flow cytometry analysis of surface markers and differentiation assays[121]; cell viability, which is usually required to be above 70%[142], can be determined through cell counting, using appropriate technology; and sterility may be ensured with microbiology testing involving bacteria, mycoplasma and endotoxin detection[88,121]. Purity and stability are paramount for

product safety and thus should be verified as well, the latter through cytogenetics assays[121] such as standard G-banding karyotype analysis[88]. Finally, potency assays are the ones that pose the biggest challenge[89,143].

Regulatory agencies such as FDA and EMA provide guidelines regarding potency assays for release testing but leave room for flexibility when it comes to selecting the appropriate test, evaluating the adequacy case by case[144]. This is due to the fact that potency measurements, which can be defined as measurements of the therapeutic activity of a drug, are designed specifically for each particular product as they must be based on the known or presumed mechanism of action (MOA) of the drug[144]. In the case of MSC-based products, due to the great variability that persists in the sourcing, manufacturing and characterization of these drugs[145], it would be useful to develop reliable, quantitative and reproducible potency assays based on cells' functionality that represent relevant biological properties of MSCs and are able to distinguish which batches are sufficiently potent, while also serving as a measure of comparability between production lots[143,144].

The development of potency assays poses a challenge for MSCs due to several factors. Firstly, MSCs are a heterogeneous population and there is a great variability between the starting material due to different sources and different donors. Secondly, as was seen previously, MSCs isolated from the source material are in short numbers and expansion is not trivial which means that the number of cells available for testing is limited as is their long-term stability. Finally, and most importantly, MSCs' MOA is not completely understood and is known to be multifactorial, which makes the task of deciding which attributes constitute sufficient measurement of potency a complex one[88,144]. This means that the potency assay or assays chosen for a specific clinical indication, since a single test will hardly be enough to predict clinical efficacy[144], is based on the pre-existent clinical data regarding the therapeutic effect of the product, and a deeper understanding of MSCs' therapeutic mechanism will mean assays that are more accurate and predictive of MSCs' function *in vivo*[88,143,144].

The currently used potency assays evaluate paracrine, regenerative or reparative mechanisms[88], as these are in the origin of MSCs' clinical efficacy. Guidelines provided by the ISCT[144] recommend the use of analytical methods, such as quantitative RNA analysis, flow cytometry or protein-based assay of the secretome, to evaluate the immuno and biochemical properties of MSCs. There is an interest in identifying surrogate markers of potency, that is, bioactive molecules that can be correlated with a certain biological activity, as these would replace the need for more complex bioassays, given that one of the previously mentioned analytical methods would suffice to detect the levels of expression of these molecules[88,143,144]. TNF receptor 1, used as a release criteria for Prochymal[88], or IDO and programmed death ligand 1 (PDL1)[146] are examples of molecules employed as surrogate markers of

immunomodulatory function in previous studies. A strategy that is also recommended by the ISCT to assess potency and that can be used to identify these molecules is the assay matrix, in which a combination of complementary analytical and biological assays that measure different attributes provides a better measure of potency[144]. An example of this is the study performed by Chinnadurai and colleagues in which the secretome and transcriptome response of MSCs to IFN-γ and peripheral blood mononuclear cells (PBMC) is analyzed and correlated with *in vitro* suppression of PBMCs by MSCs[147].

Analytical methods are useful but not enough to accurately predict potency. The immunomodulatory attributes of MSCs, important for therapeutic effect, are most commonly evaluated through functional *in vitro* assays in which MSCs are co-cultured with responder immune cells[88,143,144], normally activated T-cells[148], and where the inhibition of proliferation and cytokine production of T-cells by MSCs is measured. There are some concerns regarding the extent to which this assay is able to accurately predict *in vivo* efficacy as it is unknown if it represents the MOA of MSCs[88,144,148]. Variations of this assay have been employed including the use of unfractioned PBMC[121,147] or more purified subsets of effector cells directly involved in the disease's MOA[144]. The first option has the advantage of representing the in vivo environment more accurately than T-cells. On the other hand, it makes the assay more complex and introduces variability, as different donors will yield cells with different characteristics. The second option is bound to be more informative than the previous two and is considerably less complex. Nevertheless, it is necessary to know which disease related cell type is also affected by MSCs in order to choose the adequate effector cell[143].

As for the angiogenic properties of MSCs, these may also be evaluated in potency assays by the identification and measurement of surrogate markers such as VEGF, but also through functional *in vitro* assays like tube formation assays with HUVECs stimulated by MSCs conditioned media[88].

1.2.7. Challenges and Future Prospects

The development of MSC-based therapies has seen some advances in recent years. However, in spite of their powerful therapeutic potential, the clinical translation of MSCs is still in its first steps and there are some aspects that require further research and attention going forward.

Firstly, the biology of MSCs, responsible for their therapeutic effects, is not yet completely understood and this lack of understanding of the mechanisms of action behind the clinically meaningful activities of these cells has led to inconsistent clinical trials results which delay clinical translation[149]. In order to maximize the efficiency and therapeutic effect of MSC-based medicines, it is necessary to invest in rigorous preclinical and clinical studies to achieve a more complete characterization of MSCs, to clarify their biology, in particularly when it comes to their immunomodulatory properties, and to identify the

precise mechanisms that exert beneficial actions[26,90,150]. This will facilitate the establishment of robust and stringently defined potency assays, extremely important given the variability of the product, to select therapeutically effective batches and ensure cell quality and consistency[26,149]

Secondly, as was previously discussed, several aspects of the manufacturing process of MSCs may influence their characteristics and function[149,151]. In this regard, a prominent challenge will be to optimize and standardize the protocols used in each step of MSCs production in order to obtain a robust, comparable and sustainable manufacturing protocol[26,151], as well as to allow for more informed comparisons between different studies. Regarding clinical trials, another important challenge will be the standardization of study design, in particular regarding donor selection, cell dosage and route of administration, with the goal of, once again, facilitating the comparison among different studies[149].

Finally, despite being an important step for business model viability, cryopreservation and thawing are known to negatively affect the stability of MSC-based products, which raises economical and regulatory issues[87]. In order to ensure that thawed cells maintain the therapeutic properties and are ready for patient infusion, it will be necessary to optimize cryopreservation and thawing processes so that they are less aggressive to cells, or to establish cost effective revival strategies that allow cells to recover their normal activity. This important challenge will be further discussed in the following section.

1.3. MSCs Reviving Strategies

Cryopreservation is an important step of MSCs biomanufacturing as it constitutes an economically and logistically feasible way of having cell therapies as an off-the-shelf readily available product and opens the door to the prospect of large-scale manufacturing. Furthermore, having a cryopreserved unit as the final product allows for all quality testing to be performed prior to release and for minimal reconstitution at the moment of infusion[152].

Nevertheless, several studies report that, besides viability loses associated with the cryopreservation process, freshly thawed MSCs present impaired functional properties, namely immunomodulatory and homing capacities, which hamper their therapeutic potential and may help to explain the inconsistencies between pre-clinical and clinical trial results[153–155]. These reduced functional properties are mainly associated with the low levels of IFN-γ inducible IDO expression, potentially caused by the expression of heat-shock proteins increased by the thawing process[153], and to the disruption of the f-actin cytoskeleton which reduces cells' capacity to engraft *in vivo*[154]. Furthermore, cryoinjury may increase the risk of MSC recognition by the innate immune system, triggering an immune reaction which will hinder their beneficial performance[155].

Due to these facts, the establishment of reviving strategies to allow cells to recover their therapeutic potential before infusion has become a subject of great interest in the field of MSC therapy design. François and colleagues reported that the negative effect of thawing in MSCs' immunosuppressive properties is reversible though an acclimation period of at least 24h in culture, prior to patient administration. The efficacy of this strategy was demonstrated by the positive results obtained by Panés and colleagues in a clinical trial that used MSCs for the treatment of Crohn's disease associated complications[156], which constituted the first successful advanced clinical trial using MSCs[50], and by other studies[157]. Despite effective, this strategy eliminates the advantages that can be obtained by cryopreservation given that culturing cells prior to administration requires more manipulation and quality testing, which would amount to prohibitive costs. The question of whether cost-savings and logistical conveniences should outweigh the possible reduced therapeutic potential of the cell product is a complex one.

Considering this, there has been a considerable effort to devise other strategies to optimize the cryopreservation process in order to increase cell viability and avoid cryoinjuries. As was seen previously, several studies have focused on alternative cryopreservation solutions, namely different cryoprotective agents and reduced DMSO concentrations[137,139,140,158–160]. Another often explored strategy is MSCs priming, which means to prepare cells for a specific function through the administration of specific stimuli before freezing, such as pro-inflammatory cytokines to augment their immunomodulatory properties[161,162]. MSCs encapsulation as also emerged as not only a strategy to preserve MSCs viability and functionality during cryopreservation[163] but also as a transport solution from manufacturing site to point of care[164,165].

Regardless of the strategy used for MSC cryopreservation and transport, it is important to consider that cell viability does not necessarily correspond to cell functionality and that, despite presenting a similar phenotype to that of their fresh counter parts, cryopreserved MSCs may present reduced therapeutic properties. For this reason, it is not only important to monitor cryopreservation and thawing protocols and to optimize them, but also to perform functional assays in quality control testing, as a complement to cell characterization.

2. Materials and Methods

2.1. Human MSC Samples

Human MSC used in this study are part of the cell bank available at the Stem Cell Engineering Research Group (SCERG), iBB-Institute for Bioengineering and Biosciences at Instituto Superior Técnico (IST). MSC were previously isolated/expanded according to protocols previously established at iBB-IST[97,166]. Originally, human tissue samples were obtained from local hospitals under collaboration agreements with iBB-IST (adipose tissue: Clínica de Todos-os-Santos, Lisboa; umbilical cord: Hospital São Francisco Xavier, Lisboa, Centro Hospitalar Lisboa Ocidental, Lisboa). All human samples were obtained from healthy donors after written informed consent according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of the respective clinical institution (Carvalho, et al., 2018). Human AT-, UCM-MSC were cryopreserved with culture medium with 10% (v/v) DMSO (276855 Sigma-Aldrich) in a liquid/vapor-phase nitrogen container in 1ml cryovials (377224 Thermo Scientific) containing 500 000 cells per mL. For the purposes of this work, the following donors were used: L090724 and #14DD, isolated from AT, and #74ED and #83ED, isolated from UCM.

2.2. Cell Thawing

Vials containing cells from the selected donors, all in passage 1 were retrieved from cryostorage and thawed in a water bath (Memmert WNB / WNE / WPE) at 37°C. Thawed cell suspensions were gradually diluted in 5 mL of low-glucose DMEM (31600-091 Gibco) supplemented with 20% (v/v) FBS (10270-106 Gibco) and 1% (v/v) Antibiotic-Antimycotic (15250-062 Gibco) and centrifuged for 7 minutes at 350 RCF (ScanSpeed 1580MGR). The resulting supernatants were discarded, and pellets were resuspended in approximately 3 mL of low-glucose DMEM supplemented with 10% (v/v) MSC-qualified FBS (12662-029 Gibco) and 1% (v/v) Antibiotic-Antimycotic. Cell number and viability were determined using the Trypan Blue (15250-061 Gibco) exclusion test. Cells were seeded into necessary T-flasks (Corning) for a cell density range of 3000-12000 cells/cm^2, using MSC-certified FBS containing medium and incubated at 37°C and 5% CO_2 in a humidified atmosphere. Culture medium was changed every 3-4 days until cells reached between 70-80% confluence.

2.3. Cell Passaging

After reaching 70-80% confluency, cells had their exhausted medium removed and were washed with PBS (21600-044 Gibco). Then, in order to detach MSCs from their cultureware surfaces, cells were treated with either a solution of 0.05% (v/v) Trypsin (1590-046 Gibco) and 0.1 mM EDTA (03690 Sigma-

Aldrich) in PBS for FBS-containing conditions or with TrypLE (A12177-01 Gibco) for xenogeneic-free culture for 7 minutes at 37°C. TrypLE was previously diluted in PBS from a 10x concentrated solution. Following cell detachment, dissociation agents were inactivated or diluted by completing the working volume of each T-flask with fresh FBS-supplemented culture medium or exhausted HPL-supplemented culture medium, respectively. Cells were centrifuged for 7 minutes at 350 RCF, resuspended in their respective culture medium and counted as previously described.

To establish FBS-free conditions, MSC donors isolated in FBS-containing medium were adapted *in vitro* to xenogeneic-free culture based on HPL (HPCHXCGLS50 AventaCell) supplementation. Dissociated cells that were thawed and cultured in FBS-containing culture medium were equally split. Half of the cells were seeded in T-flasks with the same FBS-supplemented culture medium at a cell density of 3000 cells/cm^2. The other half were diluted in PBS to wash all traces of FBS, centrifuged and plated in T-flasks with low-glucose DMEM supplemented with 5% (v/v) HPL and 1% (v/v) Antibiotic-Antimycotic at a cell density of 3000 cells/cm^2. Two consecutive passages in HPL-containing medium were considered sufficient to establish xenogeneic-free cell culture.

Cells were passaged until passage 4 or 5 (in order to reach the required number of cells), after which they were detached from culture flasks and used for the cryopreservation and transport assays described below.

2.4. Cryopreservation Solution Testing

Cells from the four different donors initially thawed, each expanded in FBS and HPL-supplemented culture medium, were cryopreserved in five conditions, corresponding to different cryopreservation media. For each donor and each condition, three individual cryovials were frozen to account for 3 timepoints (i.e., 1 month, 2 months and 3 months of cryopreservation). Overall, a total of 15 cryovials were frozen per MSC donor. The cryopreservation conditions used are listed below:

1. 90% (v/v) FBS or HPL-supplemented Culture Medium (depending on the expansion medium of each donor) and 10% (v/v) DMSO;
2. 90% (v/v) FBS or HPL (depending on the expansion medium of each donor) and 10% (v/v) DMSO;
3. 90% 2-8 Cellsius (PP338-100 Protide Pharmaceuticals) and 10% (v/v) DMSO;
4. CryoStor CS5 (C2999-100ml Biolife Solutions);
5. PZerve (5720 Protide Pharmaceuticals).

For each MSC donor, 3 mL of each cryopreservation medium (1 mL per cryovial) were prepared prior to cryofreezing and stored at 4°C. Cell detachment was performed as previously described, with the

exception of the exhausted medium being stored and fresh medium being used to inactivate or dilute the detachment agents (Trypsin for cells growing in FBS-supplemented medium and TrypLE for cells growing in HPL-supplemented medium). For each cryopreservation condition, 1 mL of cell suspension with a concentration of 260 000 cells/mL was cryopreserved. Since the available amount of PZerve cryopreservation medium volume was limited, the corresponding cryovials for this condition were cryofrozen with only 0.7 mL and the number of cells was adjusted to maintain the same cell concentration. Each cell suspension was washed with PBS and was centrifuged for 7 minutes at 350 RCF. After centrifugation, each pellet was resuspended in the appropriate volume of the respective cryopreservation medium. Cells in suspension were then divided among three suitably labeled cryovials. A total of 15 cryovials for each MSC donor were placed inside a CoolCell Freezing Container (432000 Corning) and stored at -80°C for approximately 24 hours, allowing a uniform and gradual decrease of temperature at a rate of -1°C/minute. After this period of time, cryovials were rapidly moved into a liquid/vapor nitrogen storage tank.

The previously reserved conditioned medium was filtered using 0.45 µm syringe filters (SLHV033RB Millex-HV) and a 30 mL luer-lock syringe (180030 Sol-M), and stored at -20°C.

2.5. Transport Solution Testing

Cells from two MSC donors, one from AT and one from UCM, expanded in both FBS and HPL-supplemented culture medium, were placed in suspension in four different transport solutions at room temperature (RT) or at 4°C as follows:

1. Fresh Culture Medium (supplemented with FBS or HPL depending on the expansion medium of each donor) at RT and 4°C;
2. Conditioned Culture Medium (supplemented with FBS or HPL depending on the donor) at RT and 4°C;
3. 2-8 Cellsius at 4°C;
4. BeadReady (BR-MNS-01 Atelerix) with Fresh Medium and with Conditioned Medium at RT.

Cells were resuspended in 50 mL falcon tubes in solutions 1, 2 and 3 at a density of 1 million cells/ml. Conditioned culture medium was supplemented with the required amount of glucose in order to reach a final concentration of 1 g/L (thus matching the glucose concentration present in fresh medium). 2-8 Cellsius was supplemented with 1% (v/v) Antibiotic-Antimycotic and filtered with 0.22 µm syringe filters (SLGV033RB Millex GV) in order to ensure sterility. Cells were counted every 24 hours and characterization assays (immunophenotype and trilineage differentiation) were performed every 48 hours until one of the following exclusion conditions were met: cell viability lower than 70% and cell concentration recovery (calculated by dividing the obtained density by the initial one) lower than 50%.

The conditions which met these criteria were considered terminated and discarded. Supernatant samples were recovered every 48 hours and at the end of the experiment, centrifuged at 360 RCF for 10 minutes and stored at -20°C for later metabolite analysis.

For solution 4, BeadReady, six million cells from the AT donor were resuspended in either fresh or glucose-supplemented conditioned medium and encapsulated in alginate beads according to the kit's instructions provided by the manufacturer. Each kit was divided in two so that formed alginate beads could be dissociated at two different timepoints. Beads were dissociated at days 5 and 10, released cells were counted, and characterization and functional assays were performed. Medium samples were recovered every 2 or 3 days, centrifuged as described above and stored at -20°C for later metabolite analysis. This analysis was performed using the YSI 2500 Biochemistry Analyzer (Xylem).

2.6. Characterization Assays

To evaluate the identity of the MSCs during the cryopreservation and transport solutions assays, their immunophenotype was analyzed by flow cytometry to ascertain the expression of characteristic surface markers. Additionally, their multilineage differentiation potential into the adipocyte, osteocyte and chondrocyte lineages was assessed. The functionality of MSCs submitted to alginate encapsulation were also verified through a hematopoietic support assay.

2.6.1. Immunophenotype

The immunophenotype of MSCs was determined by flow cytometry, resorting to a FACSCalibur cytometer (BD Biosciences). After thawing, cells recovered from each of the cryopreservation solutions (excluding the PZerve condition due to reduced cell number) were equally divided into three 5 mL round bottom polystyrene tubes (352235 Falcon) and resuspended in 100 µL PBS. For cells from the transport solution assay, their immunophenotype was analyzed every 48 hours throughout the duration of the assay. For each condition 100 000 cells were resuspended in 100 µL PBS. To analyze cell viability, a LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit (L34974 Invitrogen) was used. Cells were incubated in the dark for 15 minutes with the amine reactive dye. Afterwards, they were washed with 2 mL PBS and centrifuged for 7 minutes at 503 RCF. To assess the presence of surface antigens, cells were resuspended in 100 µL PBS and incubated for 15 minutes in the dark with the following mouse anti-human monoclonal antibodies: CD73 FITC (561254 BD Biosciences), CD90 PE (328110 BioLegend), CD44 PerCP-Cy5.5 (560531 BD Biosciences), CD105 APC (323208 BioLegend) or FITC (ab53318 abcam), CD80 PE (305208 BioLegend), CD45 PerCP-Cy5.5 (304028 BioLegend), CD11b APC (550019 BD Biosciences), CD19 FITC (302206 BioLegend) or APC (555415 BD Biosciences), CD14 PE (301806 BioLegend), CD34 PerCP-Cy5.5 (347222 BD Biosciences) and HLA-DR FITC (307604 BioLegend). Cells were once more washed with 2 mL PBS and centrifuged using the same settings described previously.

An unstained control was prepared as a negative control in every acquisition performed. Following acquisition, data analysis was performed using the FlowJo V.10 software (BD Biosciences).

2.6.2. Multilineage Differentiation

Multilineage differentiation assays (osteogenic, adipogenic and chondrogenic lineages) were performed for UCM and AT MSCs recovered from transport solutions every 48h for the duration of the assay. For the adipogenic and osteogenic differentiation, cells from each condition were plated at a density of 3000 cells/cm^2 in 3 wells of a 12-well culture plate (Falcon) and allowed to reach 70-80% confluency. Differentiation was induced by removing the culture medium and adding either osteocyte or adipocyte complete differentiation medium containing osteocyte/chondrocyte differentiation basal medium (A10069-01 Gibco) or adipocyte differentiation basal medium (A10410-01 Gibco), supplemented with osteogenesis supplement (A10066-01 Gibco) or adipogenesis supplement (A10065-01 Gibco) and 1% (v/v) Antibiotic/Antimycotic. Differentiation medium was changed every 3 days for 14 days. For the chondrogenic differentiation, cells were counted and resuspended in the necessary culture medium in order to generate 5-10 droplets of 30 µL containing 50 000 cells each. Droplets for each condition were placed on the inner side of a petri dish lid and the closed petri dishes containing the hanging drops were left to incubate overnight at 37°C, 5% CO_2 and in a humidified atmosphere. For each condition, 2-3 droplets were placed in one well of a 24-well ultra-low attachment culture plate (3473 Corning) and differentiation was induced with the addition of chondrocyte complete differentiation medium, composed of either MSCgo chondrogenic basal medium (05-220-1B Biological Industries), MSCgo chondrogenic supplement mix (05-221-1D Biological Industries) and 1% (v/v) Antibiotic/Antimycotic, or MesenCult ACF chondrogenic differentiation basal medium (05456 Stemcell Technologies), MesenCult ACF chondrogenic differentiation supplement (05457 Stemcell Technologies) and 1% (v/v) Antibiotic/Antimycotic. Differentiation medium was changed every 4 days for 14 days.

After 14 days, cells were washed with PBS, fixed with 4% (v/v) paraformaldehyde (158127 Sigma Aldrich) for 30 minutes at RT and washed with PBS again. To assess the degree of differentiation towards adipocytes, cells were stained with 0.2% (v/v) Oil Red O (O0625-25G Sigma Aldrich) in a 60% (v/v) isopropanol (P/7507/17 Fisher Chemicals) solution (responsible for staining lipidic vesicles) and for 1 hour at RT. After incubation, cells were washed two to three times with distilled water and kept in PBS. To verify differentiation towards osteocytes, cells were subjected to an alkaline phosphatase stain (to detect alkaline phosphatase activity, which is usually high in osteoblasts) and to a Von Kossa stain (detection of calcium deposits). For that, cells were first incubated for 40 minutes at RT in a solution of Fast Violet (99-21-8 Sigma Aldrich) and Naphthol (855-20ML Sigma Aldrich), being washed with distilled water afterwards. After microscopic (Leica DMI3000 B) observation of the resulting

alkaline phosphatase stain, cells were incubated for 30 minutes with silver nitrate (85193-100ML Sigma Aldrich), washed two to three times with distilled water and kept in PBS. To verify differentiation towards chondrocytes, cells were incubated for 1 hour in an 1% (v/v) alcian blue (A5268-10G Sigma Aldrich) solution (responsible for staining glycosaminoglycans), washed two to three times with distilled water and kept in PBS. All cells were then observed under the microscope and pictures were taken (Nikon Digital Camera DXM1200F) to ascertain the differentiation capabilities of each condition.

2.6.3. Hematopoietic Support

A hematopoietic support assay was chosen to assess the function of AT MSCs used in the alginate encapsulation testing (i.e, BeadReady condition). Cultured MSCs and cells retrieved at days 5 and 11 following encapsulation were plated in 2 wells of a 12-well culture plate at a density of 100 000 cells/cm^2. One of the wells was cultured using fresh culture medium and the other with glucose-supplemented conditioned culture medium. Cells were left to incubate overnight at 37°C, 5% CO_2 in a humidified atmosphere in order to originate confluent feeder layers. Mononuclear cells (MNC) previously isolated from umbilical cord blood (UCB) were thawed in DMEM+10% (v/v) FBS and enriched for CD34$^+$ cells through Magnetic Activated Cell Sorting (MACS) using the CD34 MicroBead Kit (130-046-702 Miltenyi Biotec), according to the manufacturer's instructions. UCB CD34$^+$-enriched cells were then cultured over the previously prepared feeder layers (two wells with MSCs cultured either in fresh medium or in conditioned medium) and without the presence of MSCs (one well) for seven days in StemSpan Serum-Free Expansion Medium (SFEM) II (09655 Stemcell Technologies) supplemented with 1% (v/v) Antibiotic/Antimycotic, at a density of 30 000 cells/mL (2 mL per 12-well) and in the presence of the following human cytokines: stem cell factor (SCF) (AF-300-07 PeproTech) (64 ng/mL for wells without a feeder layer and 90 ng/mL for MSC-containing wells), FMS-like tyrosine kinase 3 ligand (Flt-3L) (AF-300-19 PeproTech) (61 ng/mL for wells without a feeder layer and 82 ng/mL for MSC-containing wells), thrombopoietin (TPO) (AF-300-18-100UG PeproTech) (80 ng/mL for wells without a feeder layer and 77ng/ml for MSC-containing wells) and basic fibroblast growth factor (bFGF) (AF-100-18B PeproTech) (5 ng/mL only for MSC-containing wells). After seven days, hematopoietic stem progenitor cells (HSPC) in each well were counted to assess proliferation. Fold increase (FI) was calculated by dividing the number of HSPCs counted in each well by the number of HSPCs originally seeded. These results were normalized by dividing each FI by the FI of the No MSC well, used as a negative control.

The immunophenotype of the freshly isolated and expanded HSPCs was analyzed at day 0 and day 7 for each timepoint by flow cytometry using previously titrated CD45RA FITC (561882 BD Biosciences), CD90 PE and CD34 PerCP-Cy5.5 mouse anti-human monoclonal antibodies, as previously described.

The clonogenic potential of HSPC was evaluated at days 0 and 7 for each timepoint. At day 0, 1 000 $CD34^+$-enriched cells in 100 µL were resuspended in 2 mL of MethoCult Classic (04434 Stemcell Technologies) medium, divided into three wells of a 24-well culture plate and left for 14 days to incubate at 37°C, 5% CO_2 in a humidified atmosphere. At day 7, at the end of the hematopoietic support assay, the procedure was repeated with 2 500 cells from each condition. After 14 days, multilineage colony-forming unit (CFU-Mix), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte, macrophage (CFU-GM) colonies were counted using a brightfield microscope (Olympus CK40). Colony number was divided by the number of seeded cells to obtain the number of colonies per seeded cell. This was then multiplied by the number of HSPC harvested at day 7. FI in the number of colonies was calculated by dividing the total colony number at day 7 by the respective of day 0.

2.7. Statistical Analysis

When more than one experimental replicates were considered, data was analyzed using GraphPad Prism 8 software (GraphPad Software) and presented as mean ± SEM.

2.8. Number of Cell Population Doubling

The number of cell population doublings (NCPD) of MSC expansion was calculated using the formula $NCPD = 3{,}322 * \log\frac{N_t}{N_i}$, described in the introduction.

2.9. Metabolic Kinetic Parameter Calculation

Regarding analysis of metabolic activity, specific glucose consumption and specific lactate production was calculated by multiplying the measured amount of glucose/lactate in each timepoint (g/L), by the total volume of culture medium present at that timepoint, thus obtaining the amount of glucose/lactate in g in each timepoint. This value was then divided by the number of cells consuming/producing those metabolites at that specific timepoint, in order to obtain the specific consumption/production in g/cell. In case of larger intervals, the mean value of the number of cells present at the start and end of that interval was used.

3. Results and Discussion

3.1. Expansion of AT and UCM-MSC in FBS and HPL-Supplemented Medium

AT and UCM-MSC expansion was performed using two different culture media, DMEM supplemented with HPL and FBS, through successive cell passaging from P1 to P4. Figure 1 represents the morphology of two of the donors used at around 60% confluence, one from each source, each expanded in both culture media. It is possible to verify that both AT and UCM-MSCs expanded in HPL present a smaller and more elongated morphology than that of their FBS-expanded counterparts. This implies that greater cell densities can be achieved at confluency, which is concordant with the generally higher number of cell population doublings (NCPD) achieved per passage in this culture medium, as can be seen in Figure 2. The NCPD is similar between P3 and P4 for MSCs expanded in both HPL and FBS-supplemented culture medium.

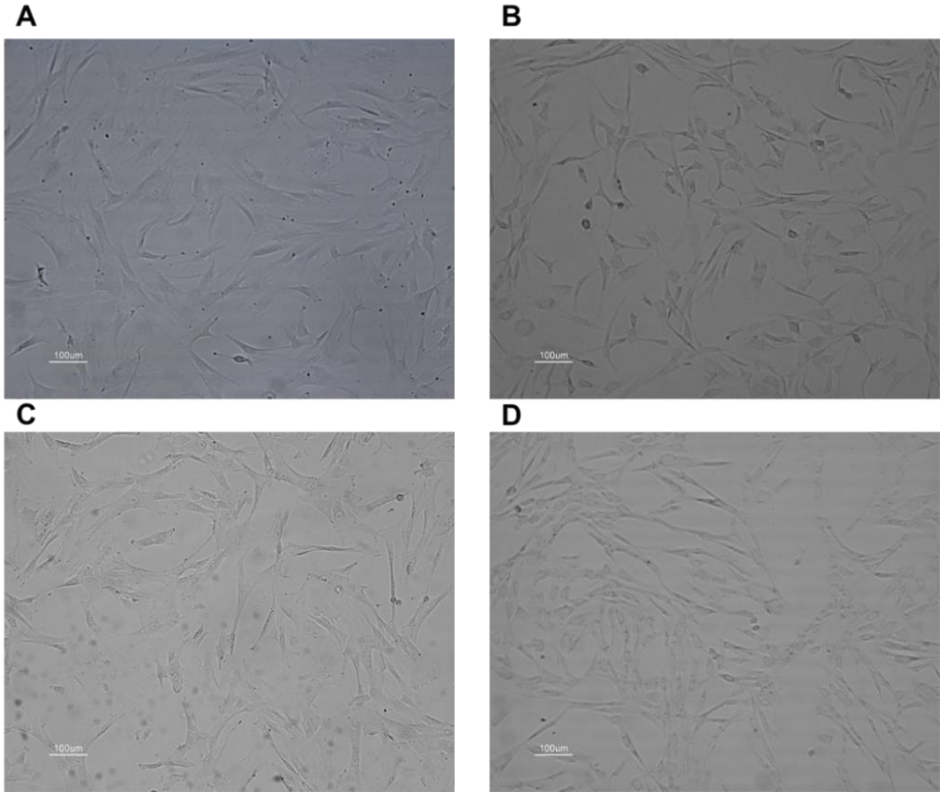

Figure 1 - Representative microscopic bright field pictures (100x ampliation) of expanded cells at P4 and 60% confluency. (A) AT L090724 expanded in FBS-supplemented medium. (B) AT L090724 expanded in HPL-supplemented medium. (C) UCM #74 expanded in FBS-supplemented medium. (D) UCM #74 expanded in HPL-supplemented medium.

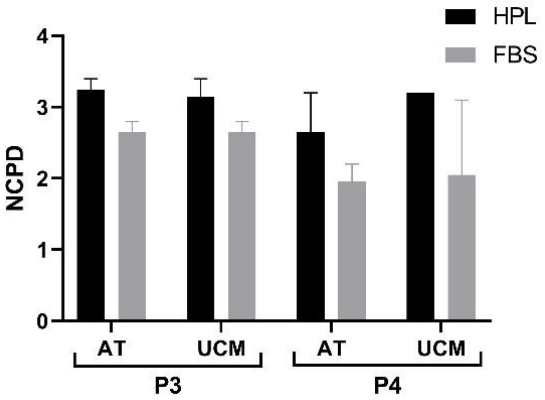

Figure 2 - Number of Cell Population Doublings (NCPD) per cell passage obtained for AT and UCM-MSC in FBS and HPL-containing growth medium (N=2). Data is represented by the mean and error bars display the SEM.

3.2. Cryopreservation Solutions

3.2.1. Cell Viability and Recovery

AT and UCM-MSCs were harvested at passage 4 and resuspended in five different cryopreservation solutions as previously described. All donors presented a cell viability over 90% at the moment of harvest, specifically with a mean cell viability (N=4) of 95.0±1.0% for AT-MSC and 92.9±0.4% for UCM-MSC. Approximately one month after each donor was cryopreserved, cells were thawed, and cell viability and recovery were calculated (Figure 3).

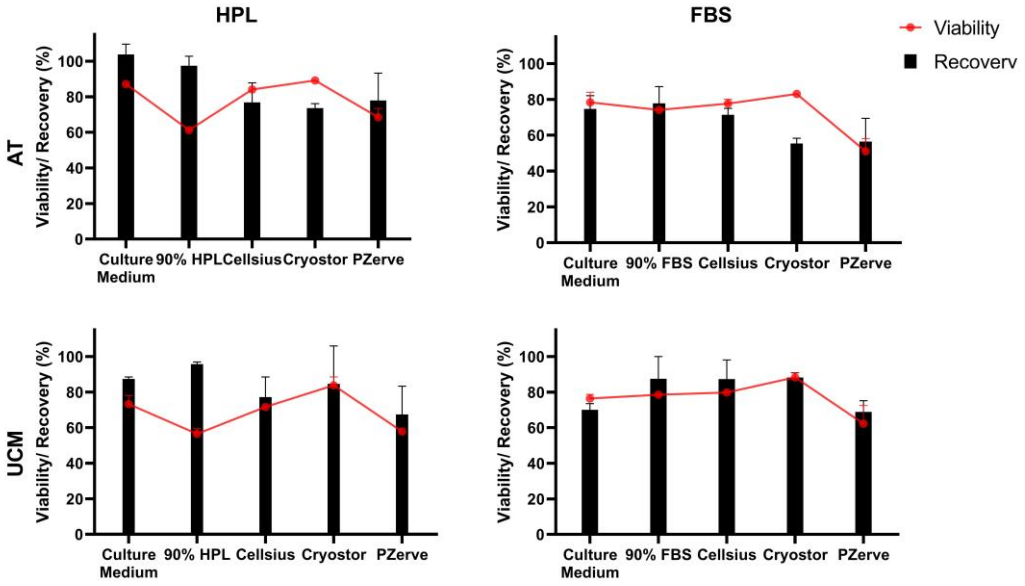

Figure 3 - Cell viability and cell recovery post-thawing at the 1-month timepoint per source and growth medium (N=2). Data is represented by the mean and error bars display the SEM.

In general, although there was no significant difference between the post-thaw viability and recovery percentages obtained for each of the cryopreservation solutions, it is possible to observe that the Cryostor condition, a commercial xenogeneic-free cryopreservation medium with 5% (v/v) DMSO, presents the highest cell viability (mean value of 86.1±1.3%, N=8). This result is in line with previously

reported results regarding this cryopreservation medium. Svalgaard and colleagues[160] obtained, for AT derived MSCs, post-thawing cell viabilities of 89.8±0.9% and 95.8±0.6% after cryopreservation with Cryostor with 2% (CS2) and 10% (v/v) DMSO (CS10), respectively. Furthermore, a cell recovery of approximately 80% obtained for both CS2 and CS10 was similar to the mean recovery percentage observed here (75.5±5.9%). Cryostor CS5 was used by Ginis and colleagues[167] who reported a mean post-thawing viability of 95.6±8.8% versus 99.5±0.5% of cultured cells in BM-MSCs, and by Gramlich and coworkers[158] who found no differences between the viability of MSCs in culture and cryopreserved with Cryostor CS5, achieving post-thawing values higher than 95%. Although these percentages are higher than the ones obtained in this case (eventually due to different cryopreservation and thawing protocols), it is possible to affirm that Cryostor CS5 does not have a particularly detrimental effect on the viability of cryopreserved cells.

Culture medium and 2-8 Cellsius, both supplemented with 10% (v/v) DMSO, follow Cryostor in the best post-thawing viability values, as these remain generally above 70% (mean values of 77.4±2.2% for FBS-supplemented medium, 80.3±3.9% for HPL-supplemented medium (N=4 in both) and 78.3±1.7% for 2-8 Cellsius (N=8)). 2-8 Cellsius is a commercially available protein-free transport medium that serves as cryopreservation medium when supplemented with 10% (v/v) DMSO. Its use in the cryopreservation of PBMC resulted in a post-thawing viability of approximately 80%[168], which is concordant with these results.

Culture medium supplemented with 10% (v/v) DMSO is considered the standard cryopreservation solution and is often used as control in cryopreservation studies. For this reason, results for this cryopreservation solution are not always presented. Nevertheless, Channadurai and colleagues[162] compared the use of different HPL concentrations in αMEM in the cryopreservation of BM-MSCs and obtained a post-thawing viability of approximately 90% following for medium supplemented with 5% (v/v) HPL and 10% (v/v) DMSO. The use of FBS-supplemented medium in these studies is more common. Fujisawa and team[139] used αMEM supplemented with 10% (v/v) FBS and 5% (v/v) DMSO as a control in their study. Moreover, Mitchel and colleagues[169] compared cryopreservation of equine BM-MSCs in MEM supplemented with 20% (v/v) FBS and 10% (v/v) DMSO with 95% (v/v) FBS supplemented with 5% (v/v) DMSO and obtained post thawing viabilities between 81 and 88%, with no significant differences between conditions. The results obtained in this experiment are in line with what is reported given that HPL-supplemented medium outperforms FBS-supplemented medium. Several cryopreservation studies, also report results using human serum albumin (HAS), a protein present in HPL and also FBS (bovine serum albumin)[170], as a cryopreservation solution. Svalgaard and colleagues[160] used a 4% (v/v) HSA solution with 10% (v/v) DMSO to freeze their MSCs and obtained

95.2±0.9% cell viability and close to 90% cell recovery post-thawing. Oja and colleagues[152] obtained 92.7-95.4% viability (higher than cultured cells) following BM-MSCs cryopreservation with HAS supplemented with 10% (v/v) DMSO. Also, Lechanteur and coworkers[121] found that BM-MSCs viability dropped from 90±4% to 76±9% following cryopreservation with 40% PBS supplemented with 40% of a 20% (v/v) HSA solution and 20% DMSO, whereas Yuan and colleagues[171] reported 88.5±0.7% post-thawing viability (higher than cultured cells) when BM-MSCs were cryopreserved in a 4.5% (v/v) HSA solution supplemented with 10% (v/v) DMSO. This alternative cryopreservation solution may be of interest for future studies.

Mean cell viability obtained for 90% (v/v) FBS was 76.3±1.4% and for 90% (v/v) HPL was 58.9±1.6%, which goes against the previously identified tendency of HPL performing better than FBS. Nevertheless, regarding post-thawing cell recovery, the 90% FBS/HPL condition presented mean values of 83.1±6.4% for FBS and 96.6±2.0% for HPL. Chinnadurai and colleagues[162] reported cell viability and recovery of approximately 90% after cryopreservation with 90% (v/v) HPL supplemented with 10% (v/v) DMSO, which differs from the obtained value for viability. This difference may be due to an overestimation of dead cells (and, consequently, an underestimation of cell viability) caused by Trypan Blue staining of debris in suspension in HPL. It is possible that, if a more accurate assay was used to assess viability, the result would be closer to what has been reported in the literature. The results obtained for FBS are in line with what has been reported in other studies. Antebi and colleagues[157] obtained 69% post-thawing viability for BM-MSCs after 7 weeks cryopreservation in 90% (v/v) FBS supplemented with 10% (v/v) DMSO and Bahsoun and coworkers[172] obtained post-thawing viability percentages similar to cultured BM-MSCs using the same cryopreservation formulation. In comparison with the commonly used culture medium condition, 90% FBS/HPL results, in general, in smaller viability percentages, although recovery rates are similar or even greater than medium for both FBS and HPL conditions.

The PZerve condition, corresponding to a commercially available DMSO and animal protein-free cryopreservation medium, presents the lowest percentages for both viability (59.9±3.3%) and recovery (67.7±5.4%), performing worse than standard culture medium. No published data regarding this cryopreservation solution could be found, although it has been used for the storage of two mast cell lines[173].

After two months of the initial cryopreservation, three donors were thawed to assess the influence of longer cryopreservation times in cell viability and recovery: AT-MSC donor L090724 expanded in FBS-supplemented medium (AT L090724 FBS), UCM-MSC donor #74 expanded in HPL-supplemented medium (UCM #74 HPL) and AT-MSC donor 14DD expanded in HPL-supplemented medium (AT 14DD

HPL). No significant difference was found between the obtained cell numbers. Figure 4 shows these results and compares them with cells thawed after a single month of cryopreservation. In general, it is possible to identify the same pattern of viability and recovery in both months which shows that one month may not constitute a relevant timeframe to observe alterations in these parameters. Ginis and team[167] thawed BM-MSCs after 1 and 5 months of cryopreservation and reported reduced post-thawing cell viability at the second time-point (71.9±8.5% at 5 months versus 95.6±8.6% at 1 month). Taking this into account, it would be interesting to assess the impact of 3 months of cryopreservation to determine if longer periods are needed.

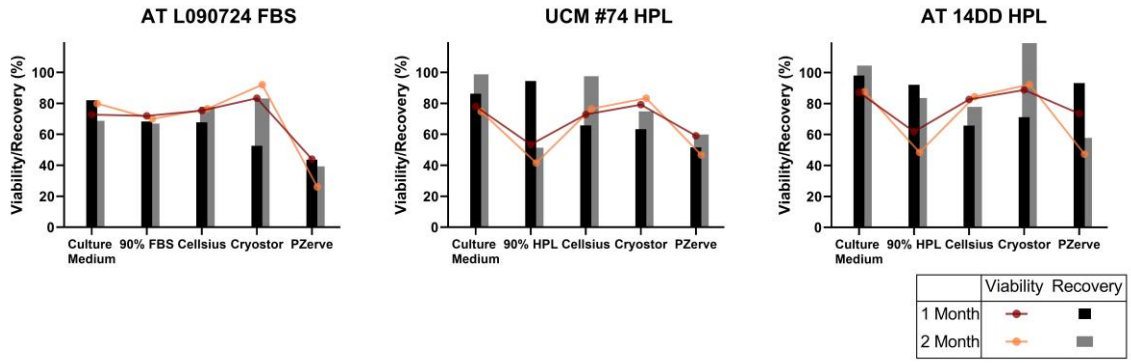

Figure 4 - Post-thawing cell viability and recovery obtained at the 1 and 2-month timepoints (N=1) for three of the cryopreserved donors.

3.2.2. Immunophenotype

To verify the identity of the thawed MSCs, their immunophenotype was analyzed by flow cytometry and compared with that of cultured cells. MSCs were considered to meet the ISCT minimal criteria if the percentage of positive cells was higher than 95% for CD44, CD73, CD90 and CD105, and less than 2% for HLA-DR, CD11b, CD14, CD19, CD34, CD44 and CD80. Figure 5 represents the gating strategy employed in the analysis of the acquired data. Firstly, a gate was used to select the desired population using parameters based on their physical properties: side and forward scatter parameters (SSC and FSC respectively). This allowed for the exclusion of cellular debris and other acellular particles from the analysis. Inside this population (termed “MSC” in Figure 5), a viability dye was utilized to identify live cells, as the presence of dead and apoptotic cells may result in unspecific staining, possibly interfering with the results. Expression of a specific surface marker was only evaluated inside this population (termed “live cells" in Figure 5). To do this, the unstained control was used as a negative fluorescence control to ascertain the presence of positive populations in the four different detection channels. The viability assay was only used in one of the three tubes of each donor.

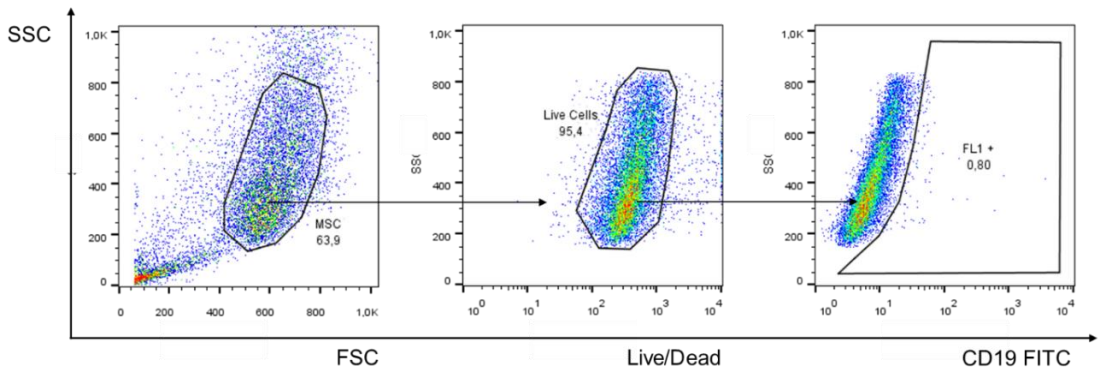

Figure 5 - Representative image of the gating strategy employed for MSC surface marker detection by flow cytometry. Firstly, a population termed "MSC" is created based on the FSC and SSC parameters. Then, a viability dye (live/dead) is used to define the "Live Cells" population. Inside this population, positive populations for each surface marker were identified using the Unstained negative control.

Results of the immunophenotypic analysis performed immediately after thawing following one month cryopreservation can be found in Figure 6. Figure 7 displays representative dot plots of the surface marker panel for post-1 month thawing for one of the AT-MSC donors cultured in FBS-supplemented medium. In this image, the expression of each cell marker is represented with the SSC denoted in the y axis and overlaid with the corresponding dot plot of the unstained control.

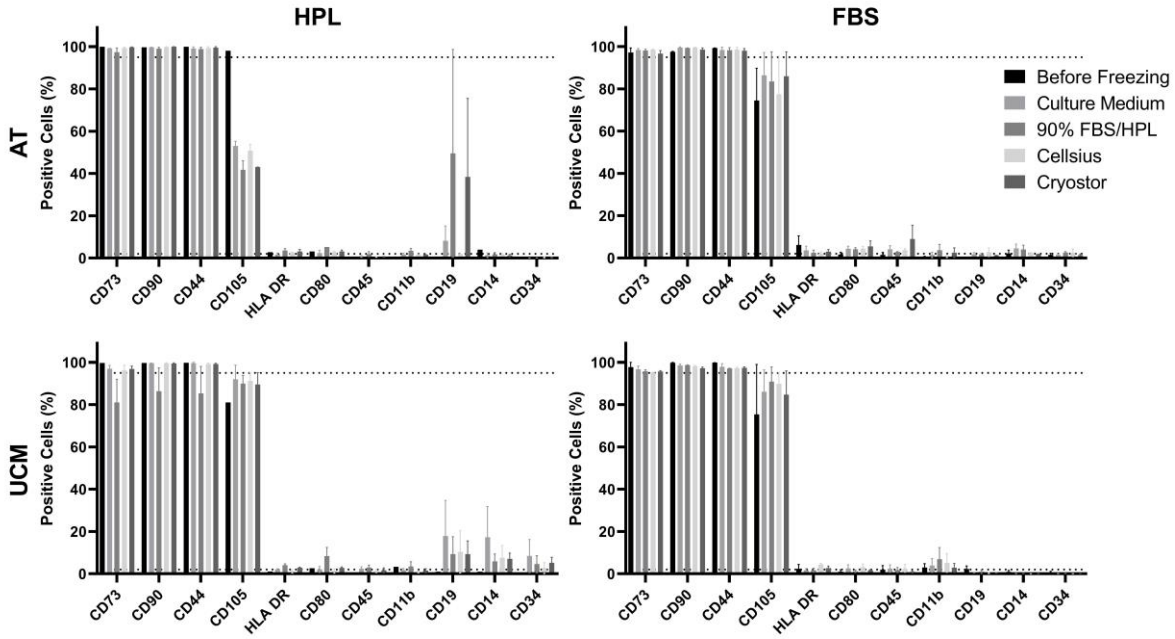

Figure 6 - Percentage of surface marker expression, before freezing and post-thawing after 1 month (N=2). Data is represented by the mean and error bars represent the SEM.

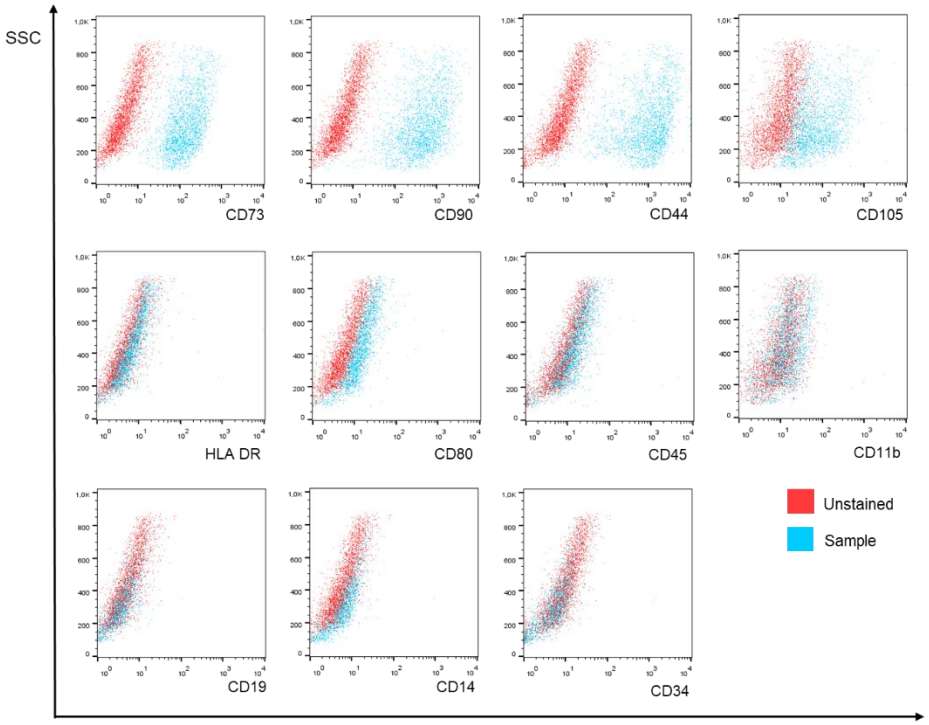

Figure 7 - Panel of dot plots representing the expression of each of the analyzed cell markers for one donor (AT L090724 FBS) post-1-month thawing, superimposed with the correspondent results of the unstained control.

Regarding the positive markers CD73, CD90 and CD44, their expression remained generally unaltered by cryopreservation, showing percentages higher than 95% of positive cells (as required by the ISCT) for all cryopreservation solutions. Several studies on cryopreservation performed immunophenotypic analysis post-thawing and concluded that the freezing process did not affect the expression of surface markers by MSCs[152,171,172,174,175]. The expression of CD105 appears to be the most affected by cryopreservation, particularly in the case of AT-MSCs cultured in HPL-supplemented medium, where the difference between the expression before and after cryopreservation is clear. Antebi and colleagues also report a decrease in CD105 expression post-thawing (91% versus more than 95% in cultured cells)[157]. The heterogeneity of CD105 expression has been previously described. Firstly, De Sousa Pinto and coworkers[110] report lower percentages of CD105 positive cells in AT-MSCs in comparison with UCM-MSCs, which is in agreement with the results obtained here. Secondly, several studies have found CD105 expression to be reversibly reduced by cell cultivation in bioreactors[110,112,115]. This CD105 downregulation has been attributed to longer exposure times to enzymatic dissociation agents and/or to higher agitation rates and consequent shear stress[107,115]. It would be of interest to plate thawed MSCs and redetermine their immunophenotype following a period in culture in order to assess if cells recovered normal expression of CD105.

The expression pattern of the negative cell markers, which are used to exclude possible contaminating cell types during MSC isolation, remained, in general, unaltered, except for an occasional increase of around 10% in the expression of CD11b, CD19, CD14 or CD80 in some cases. Some limitations may be

pointed out to the flow cytometry analysis which may shed some light on these less expected results obtained. Firstly, the analysis was limited by a reduced number of thawed cells in each condition, which resulted in a small number of events acquired (the PZerve condition was not included in this analysis due to insufficient number of cells). Secondly, only one of the three tubes analyzed per condition contained the viability dye that allowed the exclusion of dead and apoptotic cells. Given that all cell suspensions contained a considerable number of dead cells, sustained by the post-thawing cell viability percentages obtained, the non-viable cells in tubes 1 and 2 may have contributed with false positives due to unspecific staining which may have been considered in the analysis, skewing the results. Thirdly, the panel of antibodies used had not been optimized for MSCs which may have resulted in excess antibody in some cases, causing an aberrant fluorescence intensity of negative populations. Finally, the only negative control utilized was the unstained sample of MSCs. The use of a fluorescence minus one (FMO) control would have resulted in a more precise gating, by considering fluorescence interference of such a multi-color panel.

Figure 8 compares the results obtained after 1 month of cryopreservation with the post-thawing analysis performed after 2 months in three of the donors. Interestingly, when compared to one-month cryopreservation, all donors present higher than 95% of CD105 expression. Nevertheless, the lack of replicates does not allow for any definitive conclusions to be drawn. Aside from the expression of CD105, none of the donors present any relevant differences between the results from the first and second months. Ginis and colleagues[167] obtained normal immunophenotypic patterns in MSCs thawed after five months which implies once more that 2 months might not be enough to observe changes in MSC immunophenotype due to effects of cryopreservation.

In addition to an immunophenotypic analysis, most studies on MSC cryopreservation perform multilineage differentiation assays as well as post-thawing MSC seeding in order to verify the other two criteria defined by ISCT (multipotency and plastic adherence). Furthermore, as was discussed previously, impaired MSC function has been widely reported after cryopreservation, namely concerning their immunomodulatory properties. For this reason, further studies regarding the effects of the five cryopreservation solutions tested here should be considered. Both these points were investigated in the following section.

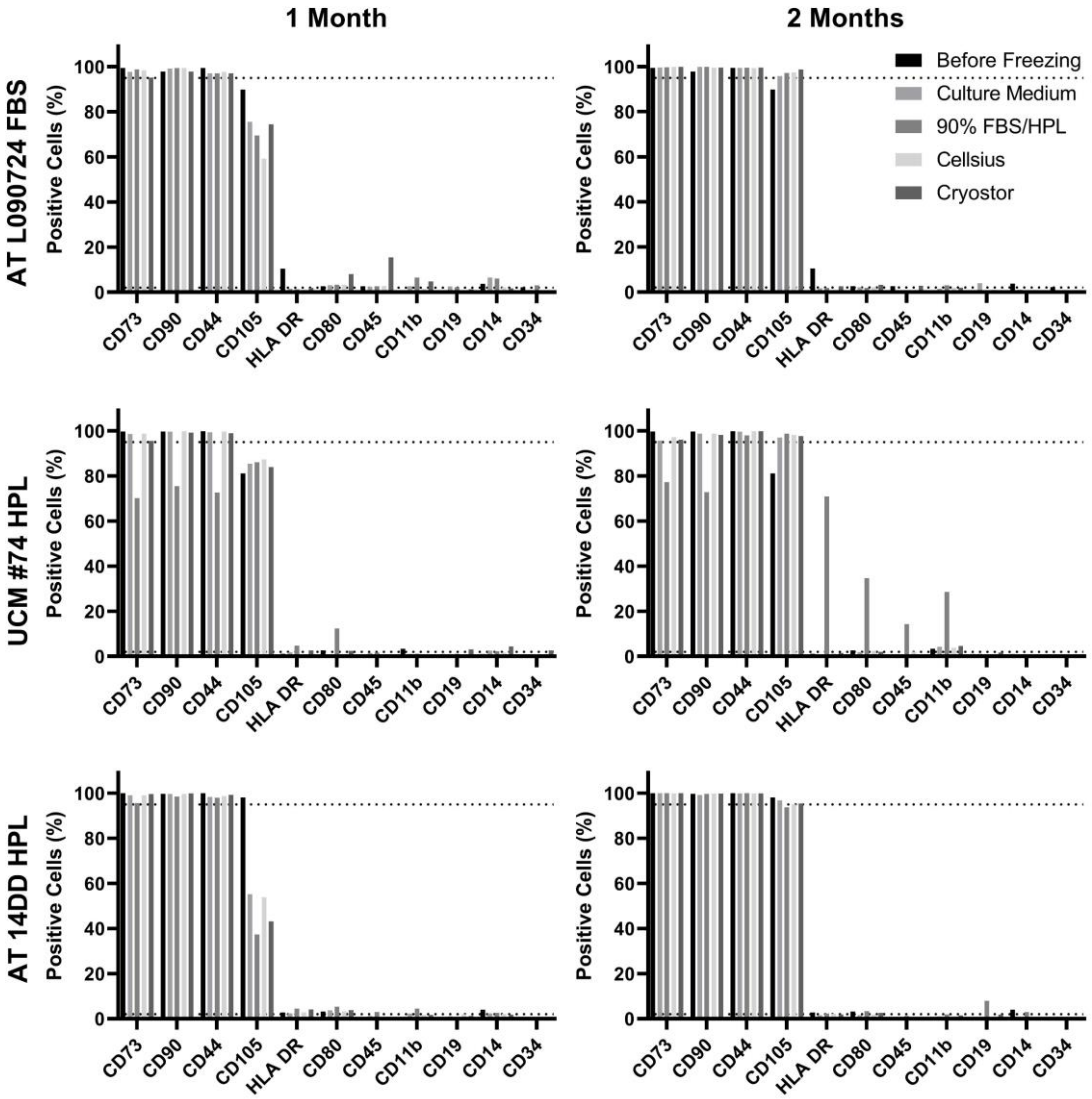

Figure 8 - Percentage of surface marker expression before freezing and post-thawing after 1 and 2 months (N=1).

3.3. Transport Solutions

3.3.1. Cell Viability and Recovery

Similarly to the cryopreservation solutions experiment, AT and UCM-MSCs were expanded during four (in the case of cells expanded in HPL-supplemented medium) or five passages (for cells expanded in FBS-supplemented medium, which have a lower expansion capacity), and placed in the five previously described transport solutions. Expanded cells from both media presented viabilities greater than 85% at the moment of harvest, with a mean viability (N=2) of 92.3±3.4% for AT-MSC and 93.5±1.3% for UCM-MSC. Unlike what was done in the previous experiment, a single donor from each source was used.

This experiment involved four conditions: AT HPL and AT FBS, consisting of AT-MSCs cultured in HPL or FBS-supplemented growth media, respectively, and UCM HPL and UCM FBS, corresponding to UCM-

MSCs expanded in the same conditions. For every donor, cells in each of the transport solutions were counted every 24 hours until the viability was lower than 70% or cell density reached half of its initial value. Cell density was used instead of the total cell number to account for the volume containing cells collected every two days to perform characterization assays. Regarding the UCM FBS condition, results are not presented given that, on day 1 of the assay, all conditions fell below the minimal criteria and were terminated. As this result was unexpected and did not agree with previously obtained results for the remaining conditions, it was not considered.

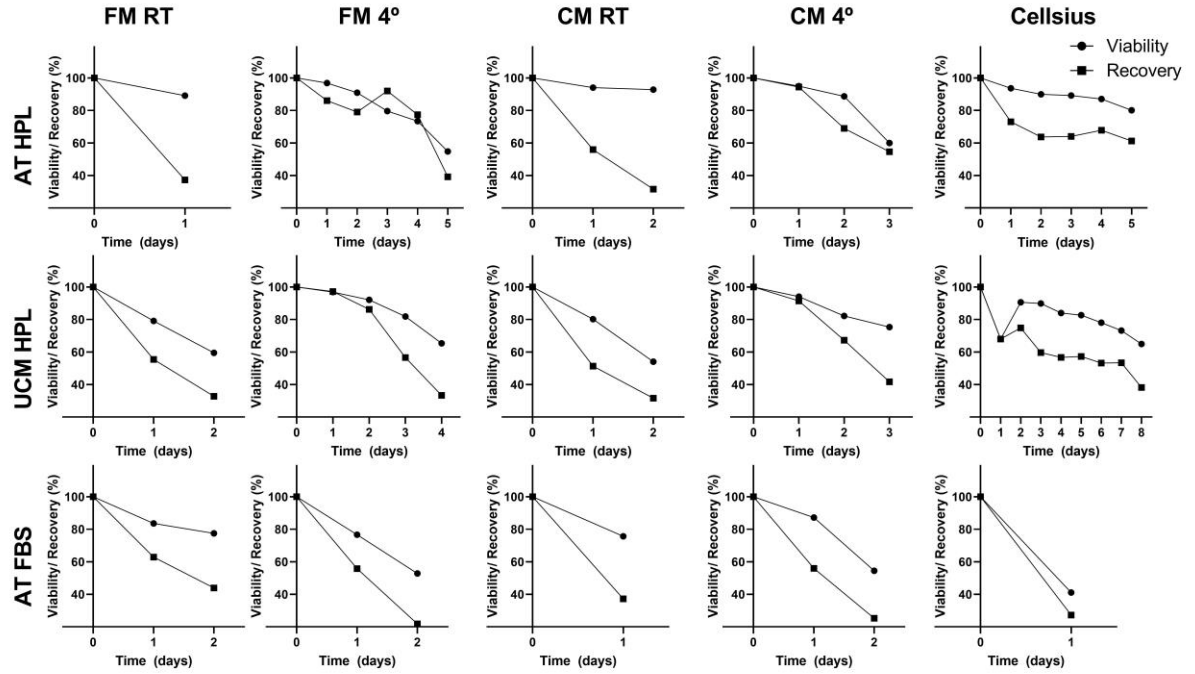

Figure 9 - Cell viability and cell density recovery calculated for each donor in each of the transport solutions every 24h until viability (calculated by dividing number of live cells by the total number of cells) was found to be lower than 70% and/or recovery (calculated by dividing the cell concentration by its initial value) was lower than 50% (N=1).

Figure 9 contains the results obtained for cell viability and recovery obtained per day for each transport solution. Regarding solutions for short term storage of cell products, these should be able to retain a satisfactory viability (the FDA defines a cell viability higher than 70% as a release criterion for cell therapy products[176]) for as long as possible so that cells can be transported during their production pipeline (*e.g.* from their manufacturing site to the corresponding point of care facility for administration). Several hypothermic preservation solutions are already available and their efficacy in MSC transport has been compared in several studies[167,177,178]. In the case of this experiment, the transport capacity of the 2-8 Cellsius solution used in the previous section was compared with MSC storage in culture medium at 4°C and at RT. Storage of MSCs in autologous conditioned medium (CM) was also evaluated, in order to determine if growth factors, cytokines, nucleic acids and other bioactive molecules secreted by MSCs could improve cell maintenance at RT and 4°C, in comparison with fresh medium (FM). MSC CM has been reported to possess anti-apoptotic, angiogenic and anti-oxidant

properties[179], making it effective in the treatment of injuries such as ischemic lesions[180], and promoting wound healing[181].

For both AT and UCM HPL, cell viability was best preserved by the 2-8 Cellsius solution, which was able to maintain MSCs above the minimum criteria for the longest time period (five and eight days, respectively). The same was not verified for AT FBS, where cells presented viability and recovery lower than 50% on day 1. Nevertheless, the lack of replicates does not allow for sound conclusions. Since no published data could be found describing the use of 2-8 Cellsius for the storage of any cell type at 4°C, experimental results could not be compared.

FM proved to be the best transport solution following 2-8 Cellsius, particularly at 4°C, where it was able to maintain MSCs with a satisfactory viability. Cells were above the criteria until day 5 in case of AT HPL and day 4 in case of UCM HPL, outlasting CM 4°C in both cases. In the case of AT FBS, both solutions performed similarly. As expected, both RT solutions were not particularly effective in maintaining MSCs, lasting only until day 1 or 2. These results are superior to those reported by Nofianti and colleagues[182] who observed the viability of AT-MSCs stored in DMEM (basal medium only) at 4°C for four days and obtained a percentage lower than 70% on day 2. In another study, Veronesi and colleagues[183] performed an 18h transport assay of BM-MSCs at 4°C using maintenance medium (composed of αMEM supplemented with 8% (v/v) HPL) and obtained a final cell death percentage of 12.13±1.58%, which is translated to a viability of approximately 87.87% and is in agreement with the results obtained for the FM 4°C condition at day 1. This study compared culture medium with a saline solution supplemented with either 1% or 5% (v/v) HSA and concluded that using medium performed better in that timeframe. Similarly, several other studies report the use of HSA as an additive for the preservation of MSCs. Pinto and colleagues[184] simulated a four hour transport of MSCs in PBS supplemented with 1% HSA at RT and 4°C, obtaining comparable viabilities (85±2% and 84±4% respectively). A similar assay performed by Celikkan and coworkers[185] yielded a 93.2±1.1% viability for UCM-MSC stored in PBS supplemented with 1% HSA at 4°C and 88.6±1.7% for cells stored at RT with the same solution, during six hours. No studies examining the hypothermic storage of MSCs in CM could be found.

3.3.2. Metabolic Activity

The metabolic activity of MSCs in the tested transport solutions was assessed every 48h or until each condition failed to meet the minimal criteria. Figure 10 shows the concentration of glucose and lactate in g/L in each transport solution through the duration of the assay. Cells belonging to the 2-8 Cellsius condition were not analyzed given that this solution does not contain any glucose.

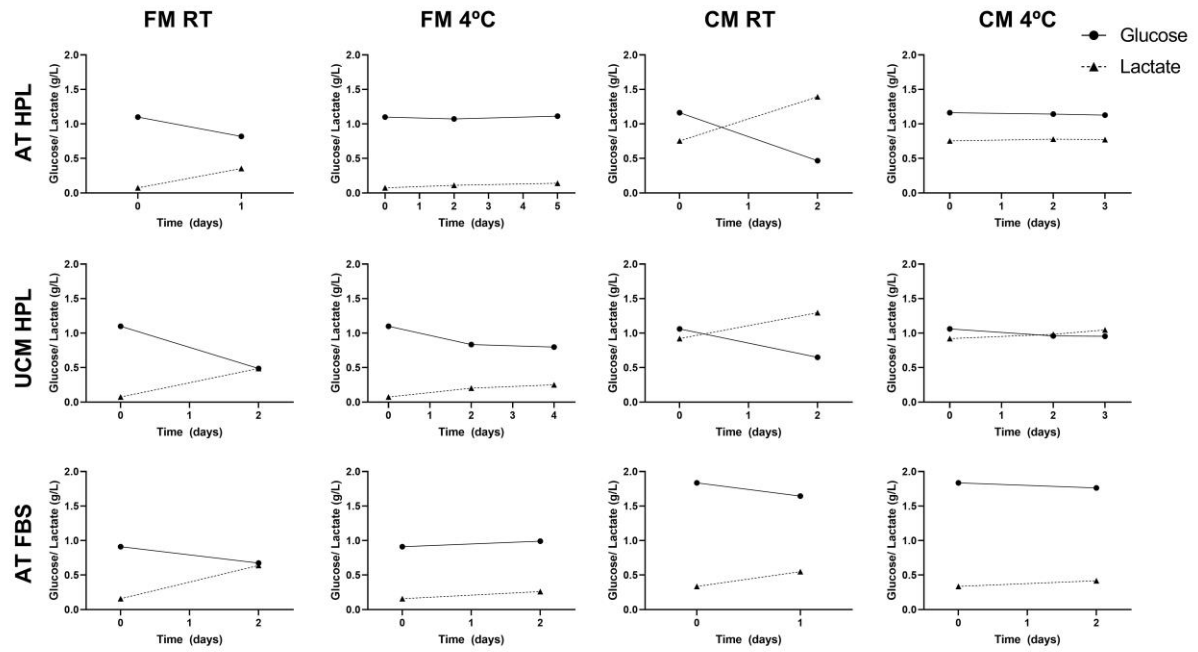

Figure 10 - Glucose and lactate concentrations (g/L) per donor for each transport solution assessed every 48h or when the condition was terminated, throughout the duration of each assay.

Interestingly, both conditions at 4°C presented very small variations in the concentrations of both metabolites through time, when compared to their RT counterparts. MSCs kept in hypothermic conditions appear to experience a reduction of their metabolic activity. This is consistent with 2-8 Cellsius being able to sustain cells more efficiently than the remaining solutions, despite not containing any glucose. Of note, glucose was not exhausted in any of the solutions, exempting nutrient limitation as a cause of assay failure. However, high lactate concentrations reached in both CM conditions may have been responsible for a poorer performance of this solution at later timepoints. This inherent disadvantage of using CM contrasts with better capacity in maintaining cell viability in the short term (day 1) (CM RT preserved AT HPL cells longer than FM RT). Yet, because the experiment was performed with limited representation (N=1), it is not possible to draw definitive conclusions from the results.

3.3.3. Immunophenotype

Once again, the identity of MSC was assessed throughout the duration of the assay resorting to the minimal criteria of the ISCT: plastic adherence, expression of a panel of surface antigens and multilineage differentiation potential. To that end, flow cytometry analysis was performed before the transport assay and every 48h in cells from all the transport solutions that fulfilled the established criteria. The same antigen panel and gating strategy (Figure 5) were employed as described previously and the results are represented in Figure 11.

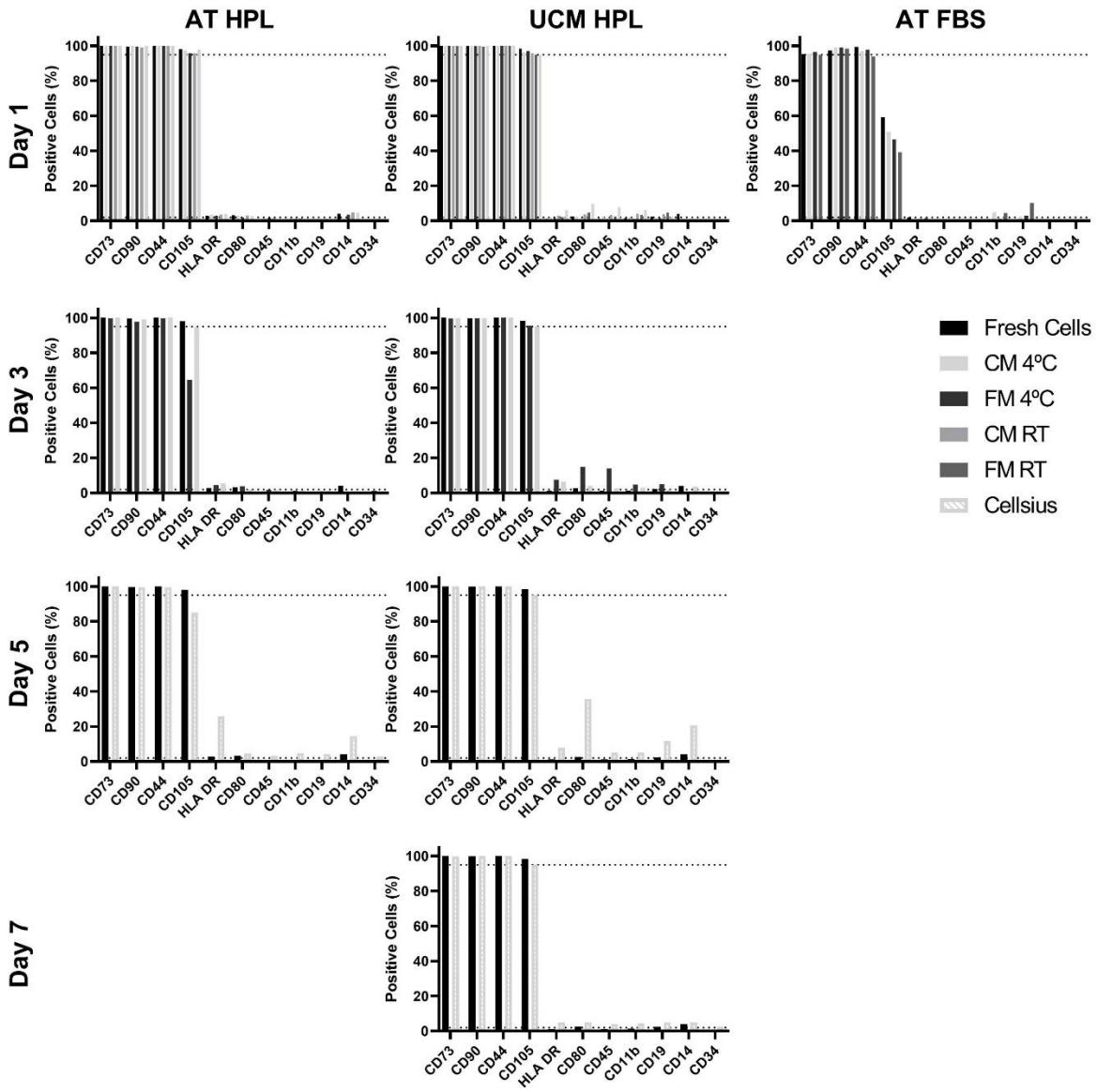

Figure 11 - Percentage of surface marker expression before transport and acquired after, every 48h, while conditions remained above the minimal acceptance criteria, for cells in each transport solution. Results per day and per donor (N=1).

On day 1 of the assay, both AT and UCM HPL conditions present a normal expression pattern, with positive markers generally above 95% and negative markers below 2% in all transport solutions. The AT FBS condition differs from this pattern only when it comes to the expression of CD105, which appears reduced. Nevertheless, this reduction was already present at day 0 so the use of transport solutions does not seem to have caused great alterations in MSC immunophenotype. As was discussed previously, CD105 is a heterogeneous marker and expressions below 95% are not uncommon. By day 3, only two conditions remained in AT and UCM HPL, namely FM 4°C and 2-8 Cellsius, whereas all conditions were terminated in AT FBS. Interestingly, the FM 4°C condition presented changes in expression pattern in both AT and UCM HPL: reduced CD105 in AT HPL and elevated HLA-DR, CD80, CD45 and CD11b in UCM HPL. Regarding the decrease in CD105 expression in AT HPL, given that this

was also observed in AT FBS and that this solution was terminated at day 5, the result could suggest CD105 expression changes are due to lower cell viability. Nonetheless, additional replicates would be required to increase the robustness of these observations. At day 5, only the 2-8 Cellsius condition remained both in AT and UCM HPL. Again, changes in MSC expression pattern appear to precede the end of the assay for a specific condition, with CD105 appearing reduced and HLA-DR and CD14 increased for AT HPL. Going against this observation, the immunophenotype of the 2-8 Cellsius on day 7 of UCM HPL returns to normal expression levels following alterations of expression of HLA-DR, CD80 and CD14 on day 5. Several 4°C transport studies, with durations ranging from six hours to seven days, using different solutions, report no immunophenotype changes[167,177,178,185]. Considering this, it is highly possible that the aberrant expression percentages found in some cases are due to the limitations of the flow cytometry analysis highlighted previously.

3.3.4. Plastic Adherence

To assess the plastic adherence of cells, MSCs were retrieved from each transport solution every 48h and plated at a density of 10 000 cells/cm^2 in T-25 flasks and incubated at 37°C and 5% CO_2 in a humidified atmosphere. All cells were able to adhere and proliferate and cell morphology was maintained in all conditions throughout the duration of the assay. Similar results were obtained by Veronesi and colleagues[183] who found MSCs maintained plastic adherence following an 18-hour incubation in αMEM supplemented with 8% (v/v) HPL at 4°C.

3.3.5. Multilineage Differentiation

Adipogenic, osteogenic and chondrogenic differentiation was initiated on day 0 and with cells retrieved from each transport solution every 48 hours, to assess if cells maintained their multilineage differentiation potential. After undergoing 14 days of differentiation, fresh MSCs (retrieved at day 0) from both sources and culture media presented calcium deposits and staining of ALP activity, characteristic of osteogenic progenitor cells, indicating the presence of an osteogenic phenotype. Nevertheless, this was more accentuated in AT-MSC-derived differentiations. Also, it was possible to identify the presence of lipidic vesicles stained by Oil Red O, indicating differentiation into adipocytes. Once again, AT-MSCs had more accentuated staining compared to UCM-MSCs. Finally, for both sources, Alcian Blue staining allowed for some staining of glycosaminoglycans in cell aggregates, suggestive of chondrogenic differentiation. No significant difference between cells from the two culture media was identified. Figure 12 is a representative picture of the multilineage differentiations performed, corresponding to the FM 4°C condition on day 1 of the assay, in both AT and UCM-MSCs. Here it is possible to verify the previously mentioned differences in staining intensity between MSCs from both sources. The same tendency was verified in the remaining days of the assay. Given that cells from conditions on day 1 and subsequent days do not present significant differences to the

corresponding pictures of MSCs in day 0, it is possible to conclude that the performed transport assay did not negatively affect their multilineage differentiation potential. The study performed by Veronesi and colleagues[183] using fresh growth medium at 4°C verified that MSCs maintained osteogenic potential after 18h in storage, which is in line with the results obtained here. Other works on MSC hypothermic storage using different transport solutions also confirm that the differentiation potential is not affected following transports for as long as 4 days[167,177,178].

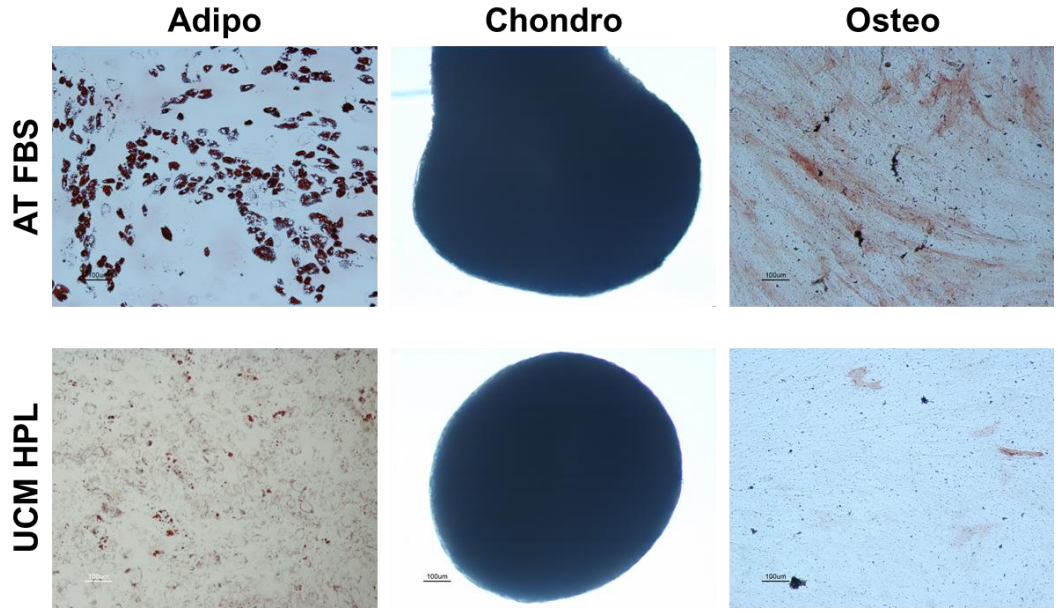

Figure 12 - Brightfield microscopic pictures (amplification of x100) of AT and UCM-MSCs corresponding to the FM 4°C condition on day 1, after 14 days of differetiation conditions to assess their multipotency. Oil Red O staining of lipidic vesicles demostrates adipogenic differentiation; Alcian Blue staining of glycosaminoglycans in MSC aggregates denotes the presence of chondrogenic phenotype; ALP activity staining highlights the presence of osteogenic progenitor cells and Von Kossa staining of calcium deposits confirms differentiation into the osteogenic lineage.

3.4. Atelerix BeadReady

3.4.1. Cell Viability and Recovery

A novel transport solution, based on cell encapsulation (*i.e.* BeadReady kit) was tested solely on a single AT-MSC donor previously used, either expanded in HPL or FBS-supplemented growth medium. Resembling the remaining transport conditions, cells were passaged for four or five passages and encapsulated according to the manufacturer's instructions, summarized in Figure 13. In total, four kits were used: for each of the growth media utilized, one kit was used for encapsulation in FM and another for encapsulation in MSC CM. Figure 14 is a representative illustration of beads formation after encapsulation and appropriate storage.

Figure 13 - Overview of the BeadReady kit encapsulation process. Briefly, cells are mixed in a sodium alginate-based component A, dropped into a calcium chloride-based gelation buffer (component B) and washed and stored in culture medium. Release is achived by adding a trisodium citrate-based dissolution buffer and the resulting suspension is centrifuged[164]. Image obtained from the BeadReady kit protocol available online on the manufacturer's website.

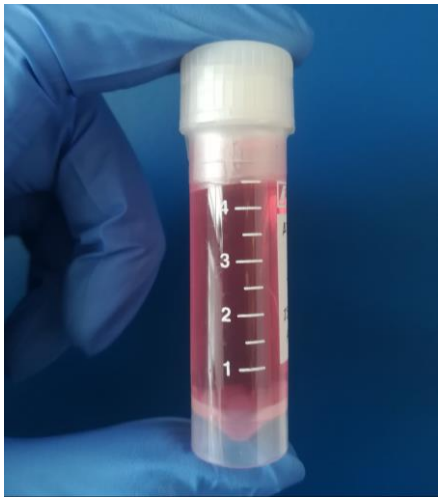

Figure 14 - BeadReady alginate beads (deposited in the bottom), 24h after encapsulation, stored in culture medium and inside a storage tube provided by the manufacturer.

As was seen in the previous sections, cryopreservation can have a detrimental effect on cell viability and post-thawing MSC functions. Hypothermic short-term storage of these cells is a viable alternative to cryopreservation, particularly in scenarios that require transportation of MSCs between two locations, but it has a short shelf life. Alginate encapsulation presents an interesting transport solution given that it involves a biocompatible polymer, amply described in the literature, and a simple and cost-effective protocol with a ready-to-use commercially available product[186]. Thus, the effects of alginate encapsulation on AT-MSC viability and phenotype were assessed and compared with the results obtained in the previous section with the remaining three transport solutions. Furthermore, an extended timeframe was considered (*i.e.* 11 days) to determine if encapsulation would be able to maintain cells viable for longer periods than the remaining solutions.

Each kit was divided in two in order to account for two time points: day 5 and day 11. Beads were stored at RT, approximately between 10°C and 20°C. At each time point, beads were dissolved and

released cells were counted, allowing for the calculation of cell viability and recovery. These results can be found in Figure 15.

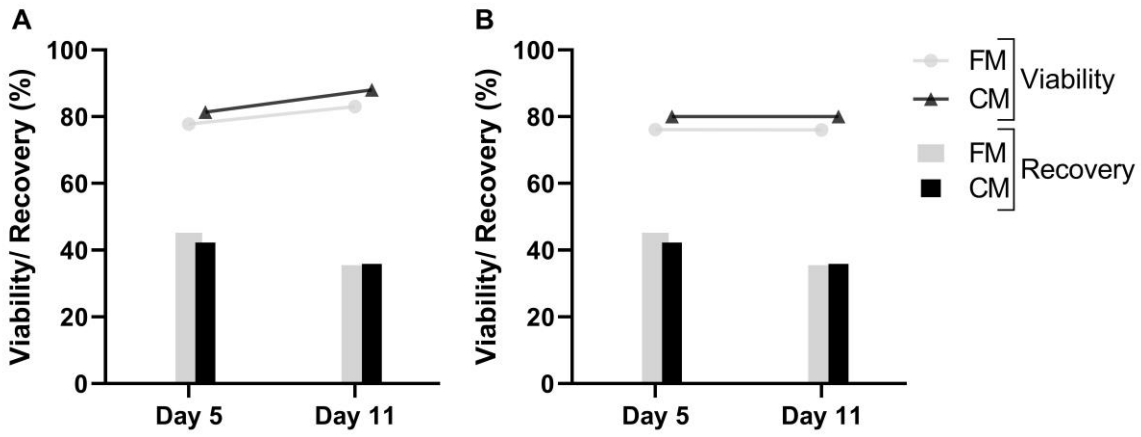

Figure 15 - Percentage of cell viability and cell recovery calculated per time point and per encapsulation condition (N=1). (A) AT HPL. (B) AT FBS.

For both culture media, encapsulation was able to maintain high cell viability percentages (mean value of 80.3±1.3%, N=8), achieving similar results for both time-points, regardless of whether FM or CM was used. This suggests that longer encapsulation periods do not affect cell viability, nor does the use of CM influence the result. These results are similar to those obtained for cell storage in the 2-8 Cellsius solution at day 5 (80.1% viability for AT HPL and 82.7% viability for UCM HPL, Figure 9). In comparison with the remaining solutions (cell storage in culture medium, either FM or CM), it is clear that alginate encapsulation preserves cell viability more efficiently and for longer periods. The results obtained are also in line with what has been reported in other studies involving alginate encapsulation. Swioklo and colleagues[187] encapsulated AT-MSC and stored them for 72 hours. This study concluded that 15°C was the ideal storage temperature, obtaining a post-release viable cell recovery of 86±6%, versus 63±5% obtained for the non-encapsulated control. Al-Jaibaji and coworkers[188] tested two storage temperatures (4°C and 15°C) in the encapsulation of multipotent adult progenitor cells and the viable cell recovery percentage attained was 66% for both conditions, superior to the 44% obtained in the control condition. Another assay by the same group[165] achieved a 77.0±2.9% viable cell recovery, following 72 hours of storage at 15°C of AT-MSCs. Finally, in a study performed by Damala and colleagues[164] using human limbus-derived MSCs, two storage temperatures (RT and 4°C) and two transport durations (three and five days) were tested. The best results were achieved by the RT condition, with 82.45±0.87% and 76.96±1.89% viable cell recovery for three and five days respectively. The viable cell recovery percentage obtained for three and five days during storage at 4°C was 65.19±1.19% and 64.45±0.81% respectively. At both storage temperatures, cell viability recovery percentage obtained for non-encapsulated control did not exceed 5%. Results obtained in this study and the data reported in the literature indicate that alginate encapsulation protects MSC viability

during storage. This has previously been attributed to the ability of alginate to stabilize the membrane of cells in suspension and to protect them from osmotic shock and mechanical stress[187].

Results for cell recovery were also similar in both culture media, remaining below 50% for both time points (mean value of 39.7±1.1%, N=8). Since the cell recovery percentage did not change from day 5 to day 11, it suggests that encapsulation time has no influence on cell recovery.

In order to investigate the initial high cell loss (higher than 50%) between encapsulation and day 5 (Figure 15), a fifth kit was once again divided in two was used to determine the cell recovery percentage immediately after encapsulation and following 5 days. A cell viability of 66.3% and a cell recovery of 52.1% were obtained after encapsulation. After five days, cell viability was 87.7% and cell recovery was 56.3%. The presence of non-encapsulated cells was searched in the washing media used during encapsulation, the dissolution buffer and the culture medium in which cells were stored, with none of these solutions containing any cells. This result indicates that the low cell recovery percentage obtained in every encapsulation was due to an initial low encapsulation efficiency and not related to the ability of the alginate beads to maintain MSCs. Assuming that the cell number obtained at day 5 in each condition is similar to the number of cells encapsulated at day 0, as was verified in the abovementioned assay, it was possible to estimate the encapsulation efficiency of the Beadready kit by dividing the sum of the number of live and dead cells by the number of cells intended for encapsulation: 58.0±4.8% (N=5, mean±SEM). Dead cells were also considered in this calculation given that the pre-encapsulation cell viability was high, and the dead cells obtained were most likely counted for encapsulation.

3.4.2. Metabolic Activity

The metabolic activity of encapsulated cells, namely their glucose consumption and lactate production, was evaluated by analyzing culture medium samples in which beads were suspended throughout the duration of the experiment, as was previously described. These results are summarized in Figure 16. Once again, there is not a significant difference between encapsulation in FM and CM, concerning their metabolic activity. Interestingly, AT HPL cells present a dynamic metabolic activity with a biphasic behavior, since they appear to have a more accentuated consumption of glucose and production of lactate between day 0 and day 5 than in the remaining days of the assay. Comparatively, AT FBS cells present low and a more constant metabolic activity along the different timepoints, with very small changes in the concentration of metabolites between days 0 and 11. Specific glucose consumption and specific lactate production were calculated as previously described regarding three time periods, namely days 0-5; days 5-11, and between days 0-11 (Table 6 and Table 7).

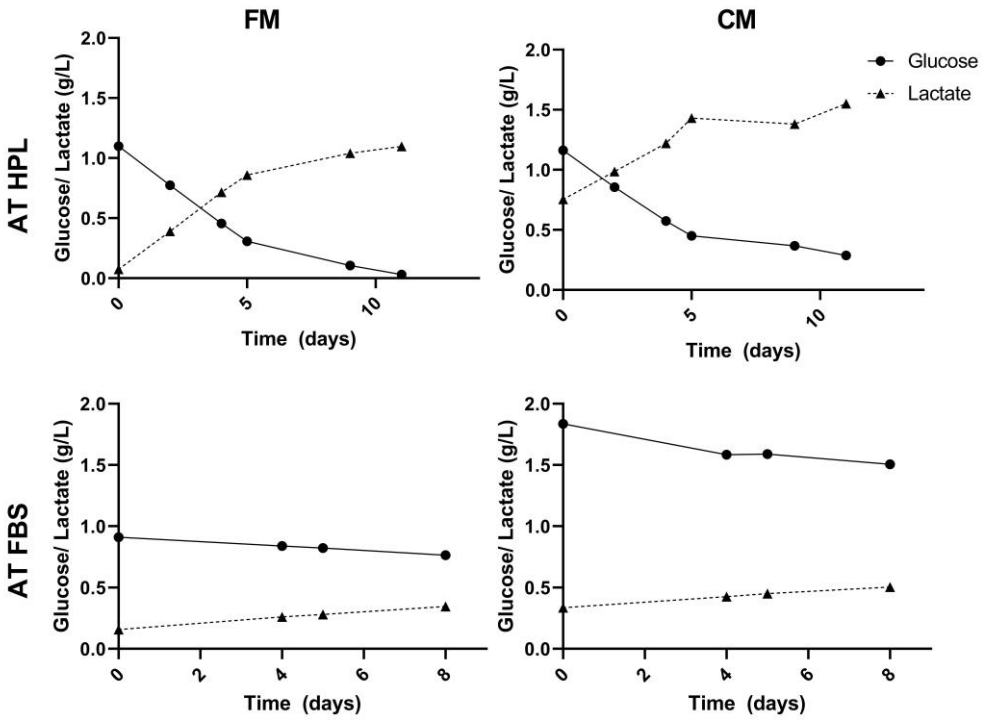

Figure 16 - Glucose and lactate concentrations (g/L) per culture medium throughout the duration of each assay.

Table 6 - Glucose specific consumption rate obtained by dividing the glucose consumed (g) in each time interval by the number of cells present at that timepoint. Data presented in g/cell.

	AT HPL		AT FBS	
	FM	**CM**	**FM**	**CM**
day 0 – day 11	24.36 E-10	20.38 E-10	6.95 E-10	14.92 E-10
day 0 – day 5	16.96 E-10	16.85 E-10	5.79 E-10	13.47 E-10
day 5 – day 11	6.08 E-10	3.03 E-10	1.28 E-10	1.17 E-10

Table 7 - Lactate specific production rate obtained by dividing the lactate produced (g) in each time interval by the number of cells present at that timepoint. Data presented in g/cell.

	AT HPL		AT FBS	
	FM	**CM**	**FM**	**CM**
day 0 – day 11	22.10 E-10	16.01 E-10	8.86 E-10	7.61 E-10
day 0 – day 5	13.38 E-10	10.14 E-10	5.51 E-10	4.39 E-10
day 5 – day 11	7.32 E-10	5.48 E-10	3.41 E-10	3.20 E-10

Analyzing the specific metabolic activity of each condition, it is possible to confirm the previous observation that consumption/production of metabolites was indeed more accentuated in the first five days of the assay and that neither the culture medium volume nor the number of cells influenced this fact. The analysis of specific consumption/production of metabolites between days 0 and 11 confirms the initial observation that the metabolic activity is indeed different depending on the expansion media. More replicates would be necessary in order solidify these observations. Because the metabolic activity of cells can be informative regarding cell fitness, Swioklo and colleagues[187] analyzed the metabolic activity of cells post release from encapsulation and concluded it was not

affected. Lack of studies concerning the analysis of the metabolic consumption/production during a transport assay highlight the novelty of these experiments.

3.4.3. Immunophenotype

In each timepoint, following beads dissolution, the identity of the obtained cells was evaluated by verifying the three ISCT criteria, as what was done for the remaining transport solutions. The results of the immunophenotypic analysis can be found in Figure 17.

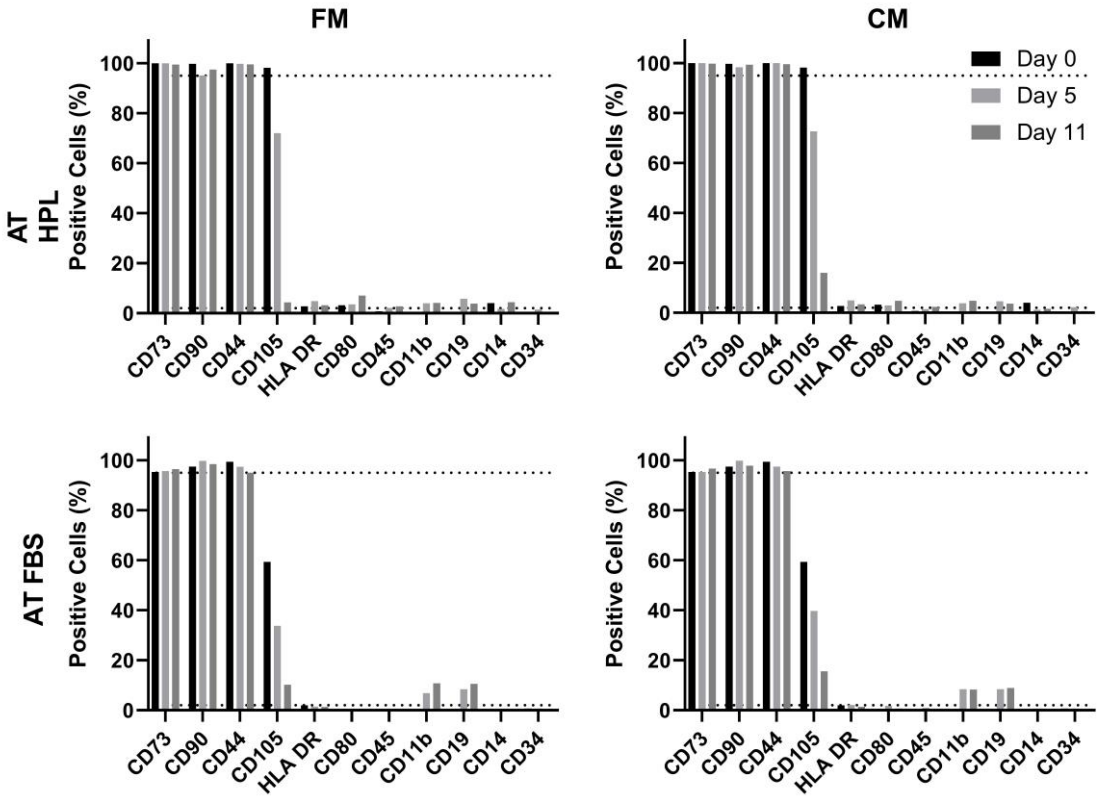

Figure 17 - Percentage of surface marker expression acquired at day 0, before encapsulation, and at days 5 and 11, post-beads dissolution, for AT HPL and AT FBS (N=1).

The expression of the positive markers CD90, CD73 and CD44 was not affected by encapsulation in any of the release days for any of the conditions. This is in line with results of previous sections. Similarly, the negative markers HLA-DR, CD80, CD45, CD34 and CD14 remained generally unaltered by the assay. This result agrees with what was obtained by Al-Jaibaji and colleagues[165], who found alginate encapsulation did not affect cells immunophenotype. Similarly, Swioklo and team[187] found CD45, CD14 and HLA-DR negative markers to be unaffected by the transport solution. However, the reduction in expression of CD90, CD73 and CD44 described by this study was not observed here.

The expression of CD11b and CD19 appears elevated following release at days five and 11 in AT FBS condition. This increase in expression did not exceed 10% and is similar to what was obtained in previous flow cytometry analysis, limitations of which were discussed above. In the four conditions it

is also possible to verify that the expression of CD105 decreases with encapsulation time. This cell marker was not investigated in other studies, making it hard to compare. These changes may be related with the previously described heterogeneity of CD105.

3.4.4. Plastic Adherence

Regarding plastic adherence, AT-MSCs from both culture media were able to adhere, proliferate and maintain morphology after bead dissociation when plated at a density of 10 000 cells/cm^2 in T-25 flasks and incubated at 37°C and 5% CO_2 in a humidified atmosphere. This was verified in all timepoints and agrees with the results obtained in other studies. Swioklo, Al-Jaibaji and Damala and their teams all report cells were able to adhere post-release from encapsulation and proliferate maintaining a normal MSC morphology[164,165,187,188]. These results indicate that alginate encapsulation does not compromise MSCs proliferation potential.

3.4.5. Multilineage Differentiation

Finally, concerning multilineage differentiation potential, adipogenic, chondrogenic and osteogenic differentiations were initiated for AT HPL and AT FBS cells at day 0 and post-bead dissolution at days 5 and 11. After 14 days under differentiation conditions, AT-MSCs from both growth media presented phenotypic characteristics indicative of differentiation into each of the three lineages: lipidic vesicles stained by Oil Red O, glycosaminoglycans stained by Alcian Blue and calcium deposits and ALP staining activity, corresponding to adipo, chondro and osteogenic differentiation, respectively. No significant difference was found between AT-MSCs cultured in FBS or HPL-supplemented medium, nor between cells encapsulated in FM or CM. Since the differentiation potential of cells released at days 5 and 11 was found to be similar to that of fresh cells, alginate encapsulation was proven to not affect the multipotency of AT-MSCs. This is concordant with the result obtained by Swioklo and colleagues[187], where MSCs maintained their differentiation potential into the three lineages following 72 hours encapsulation in alginate. Figure 18 is a representative picture of stained AT HPL MSCs from day 5 of encapsulation, acquired following 14 days of differentiation. A picture taken with larger amplification highlights the stained lipidic vesicles in more detail.

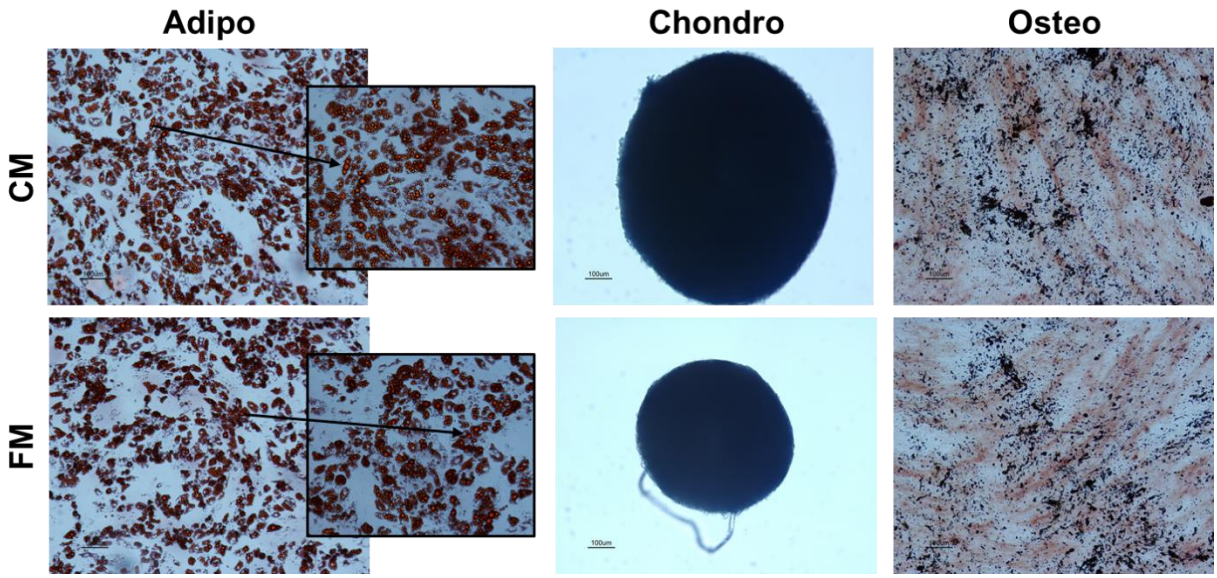

Figure 18 - Brightfield microscopic pictures of AT HPL cells at day 5, after 14 days under differentiation conditions into the adipogenic, chondrogenic and osteogenic lineages. Oil Red O staining of lipidic vesicles demostrates adipogenic differentiation; Alcian Blue staining of glycosaminoglycans in MSC aggregates denotes the presence of a chondrogenic phenotype; ALP activity staining highlights the presence of osteogenic progenitor cells and Von Kossa staining of calcium deposits confirms differentiation into the osteogenic lineage. Amplification of 100x in all pictures except smaller adipogenic pictures taken with 200x.

3.4.6. Hematopoietic Support

Although the expression of a panel of surface antigens, plastic adherence and multilineage differentiation potential are sufficient criteria to attest the identity of MSC as a quality control measure, the maintenance of MSC function is also relevant to assess. To that end, AT-MSCs retrieved from encapsulation were submitted to a hematopoietic support assay. Feeder layers of MSCs from the FM and CM conditions were established on days 0, 5 and 11 of encapsulation for both culture media and their ability to support the proliferation of HSPC was compared among them and with HSPC proliferation in the absence of stroma. A representative picture of the three conditions plated can be found in Figure 19.

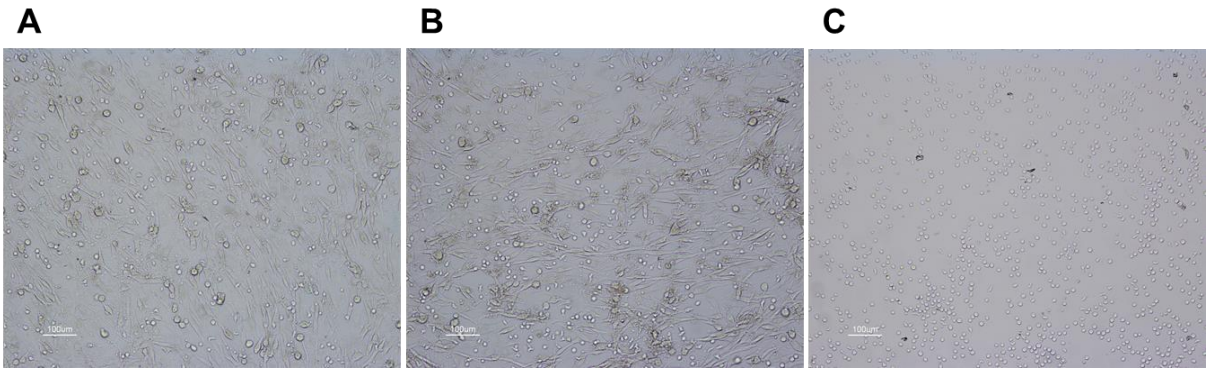

Figure 19 - Brightfield microscopic picture of a coculture of AT-MSCs and HSPCs on day 3 (100x amplificaton). (A)AT-MSC cultured in Fresh Medium. (B) AT-MSC cultured in Conditioned Medium. (C) HSPC with no stroma.

The ability of each condition to support the *ex vivo* expansion of HSPC was evaluated by the fold increase in total number of cells plated. Figure 20 highlights that after 7 days of coculture, $CD34^+$-enriched cells were able to proliferate more efficiently in the presence of an MSC feeder layer, as the fold increase in $CD34^+$ enriched cell number is always more elevated in FM and CM conditions than it is for when no stroma is present. This is true for MSCs cultured both in HPL and FBS-supplemented medium and for the three time points of the transport assay. Once again, it is also clear that no significant difference can be found between CM and FM conditions in any of the assays. To better compare the results per timepoint, the fold increase of each condition was normalized by dividing its value by the negative control (*i.e.* absence of MSC feeder-layers). A reduction in the ability to support HSPC expansion in post-encapsulation MSCs was observed for AT HPL, when compared with fresh cells. However, there is no difference between performances of MSCs from day 5 and day 11 of the transport assay, which suggests that even though this function is affected by encapsulation, the duration of the encapsulation period does not play a part in this outcome. In the case of AT FBS, there is no significant difference between the expansion of HSPCs on days 0 and 5, which goes against the tendency found in AT HPL. Furthermore, the fold increase of HSPC number on day 11 is closer to that of the negative control than in the remaining days, hinting to a loss of support capability by MSCs. The fact that all hematopoietic support assays were performed using $CD34^+$-enriched cells from the same umbilical cord donor except for AT HPL on day 0, may help to explain the abovementioned results.

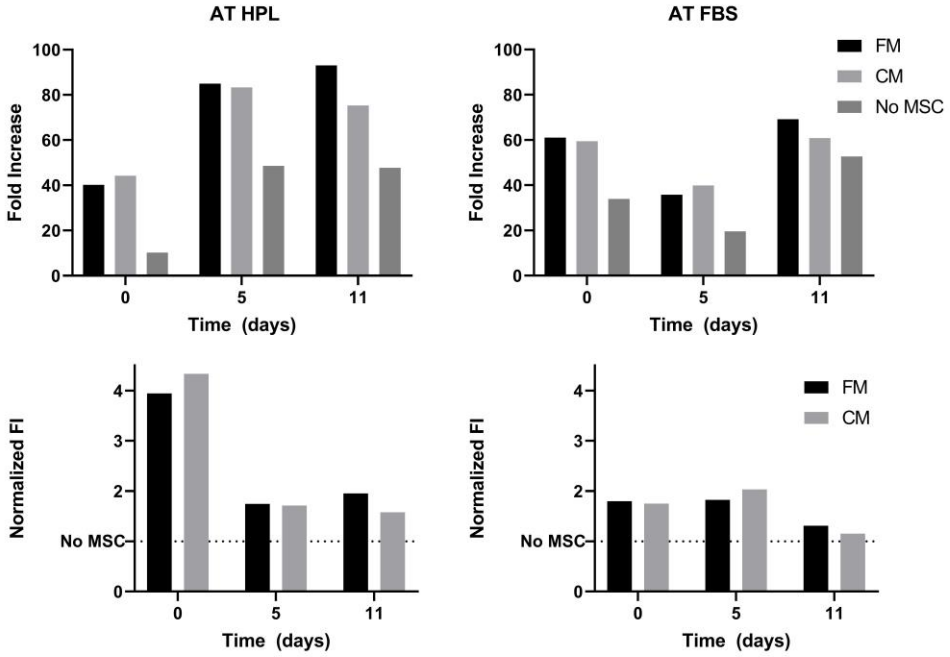

Figure 20 - Fold increase in HSPC number after 7 days culture with and without MSCs retrieved from encapsulation at days 0, 5 and 11. The three experimental conditions considered were MSCs cultured in FM and CM and No MSCs. Normalized FI was obtained by dividing the FI of each condition by the respective No MSC FI.

Besides the fold increase of total number of seeded cells, the fold increase of more primitive populations was also analyzed. CD34 is a surface antigen expressed by HSPC and its co-expression with CD90, combined with the absence of expression of CD45RA constitutes a characteristic phenotype of more primitive hematopoietic stem cells. Thus, the immunophenotype of HSPCs pre- and post-expansion was evaluated by flow cytometry using the antibodies CD34, CD45RA and CD90 and the fold increase in $CD34^+$, $CD34^+CD45RA^-$ and $CD34^+CD45RA^-CD90^+$ populations was determined. Figure 21 illustrates the gating strategy used in the analysis of the flow cytometry data acquired regarding HSPCs.

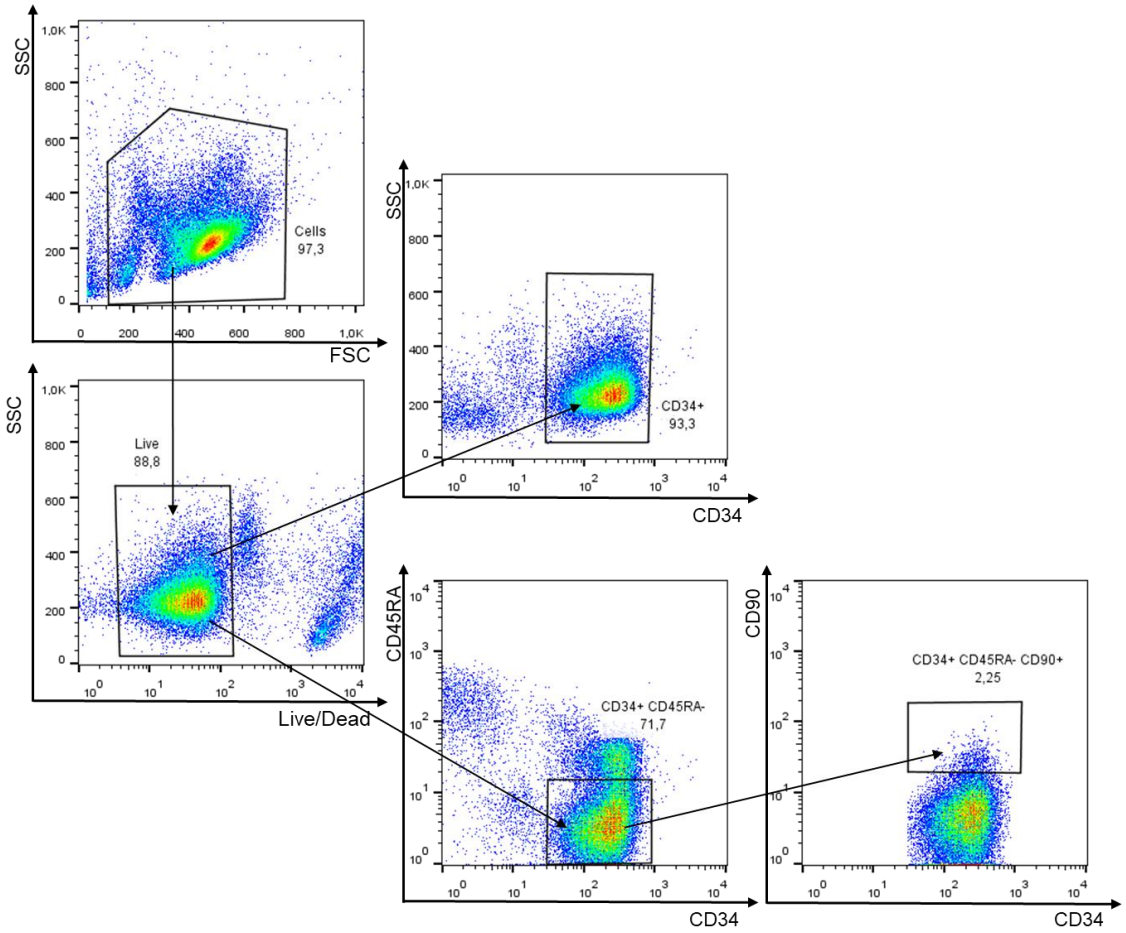

Figure 21 - Representative image of the gating strategy employed for HSPC surface marker detection by flow cytometry. Firstly, a population termed "Cells" is created based on the FSC and SSC parameters. Then, a viability dye (live/dead) is used to define the "Live" population. The co-expressions of each surface markers are determined (gates "$CD34^+$", "$CD34^+CD45^-$" and "$CD34^+CD45^-CD90^+$").

The results of this analysis are summarized in Figure 22. Even though the percentages of the analyzed populations decreased with time in culture (data not shown), there is an increase in the absolute number of these cells, denoted by the fold increase. In all six hematopoietic support assays performed it is possible to verify that this fold increase was always more accentuated in the FM and CM conditions, suggesting the presence of MSC feeder layers allowed for a more effective maintenance of primitive populations during HSPC expansion. Similar results have been reported in other studies involving hematopoietic support assays with MSCs[189,190]. Once more there is no clear difference between the

results obtained for CM and FM conditions and the longer periods of MSC encapsulation do not present a clear pattern of influence over the hematopoietic support ability of these cells.

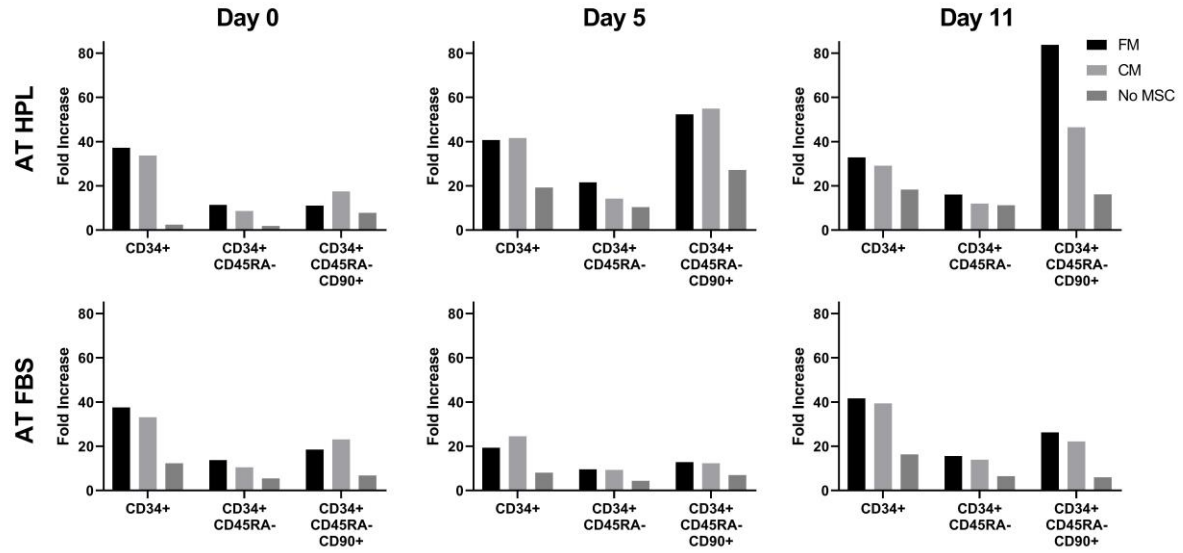

Figure 22 - Fold increase of different HSPC subpopulations expressing the analyzed phenotypes (obtained by multiplying the percentages of each population at each timepoint by the corresponding number of cells present and then dividing the number of cells per population at day 7 of the assay by its correspondent at day 0), acquired before the transport assay (day 0) and following each of the four encapsulation assays performed (days 5 and 11 using AT HPL and FBS cells), (N=1).

Finally, HSPCs from each of the assays performed where characterized in terms of their clonogenic potential. The number of BFU-E, CFU-GM and CFU-Mix generated was determined before and after HSPC expansion and the fold increase in the number of colonies was determined as previously described. The variation in total percentage of each colony type pre- and post-expansion was also calculated. These results are presented in Figure 23 and Figure 24.

In general, the results obtained are heterogeneous and it is difficult to discern a clear tendency regarding the possible effects of encapsulation in this assay. Possible inconsistencies at cell seeding and eventual colony counting errors caused by high colony density may have also had influence on the quantification of this assay. In general, the FI in CFU-GM number is more significant in FM and CM conditions (containing MSCs), whereas FI in CFU-Mix number is more notorious in HSPCs expanded in the absence of MCSs. This is also reflected in the percentages of both colony types post-expansion in all conditions. Nevertheless, regarding the fold increase in total number of colonies, there is no significant difference between the results obtained for the absence of stroma conditions and the MSC-containing conditions of FM and CM, in all timepoints and in both culture media (except for AT HPL day 0). Given that other studies report a higher ability to maintain clonogenic potential in HSPCs expanded in the presence of MSCs[189], it is possible that alginate encapsulation may cripple this capacity.

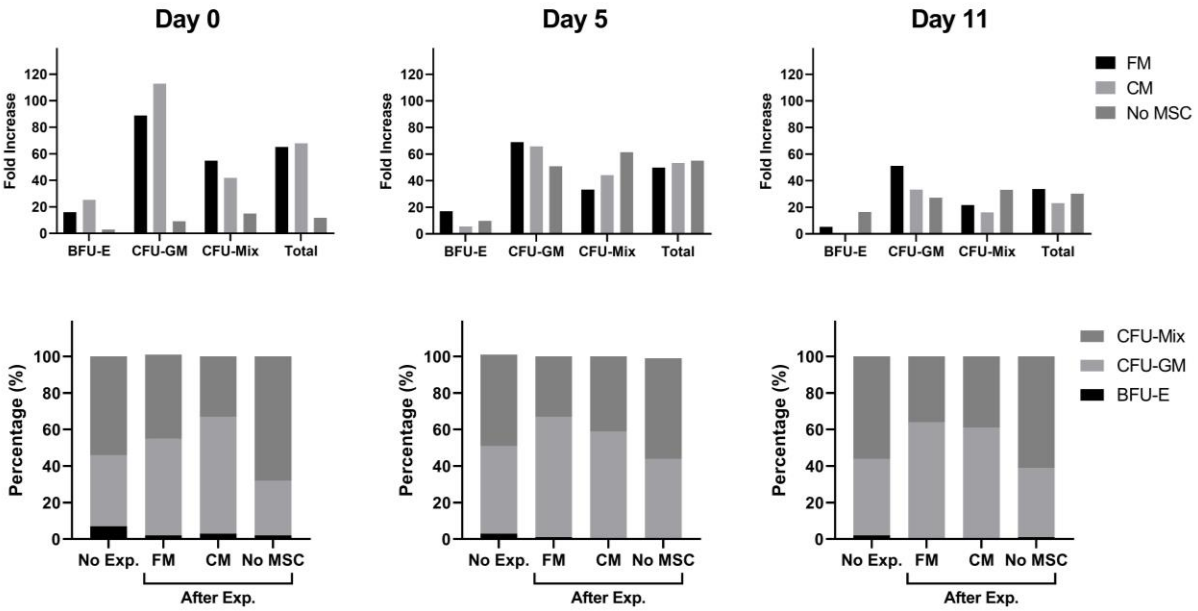

Figure 23 – Data regarding AT HPL. On top, fold increase in number of BFU-E, CFU-GM and CFU-Mix and total fold increase in the clonogenic potential of expanded HSPCs resulting from the hematopoietic support assays performed at days 0, 5 and 11 of the transport assays. At bottom, percentages of each colony type obtained before and after HSPC expansion, for each of the hematopoietic support assays.

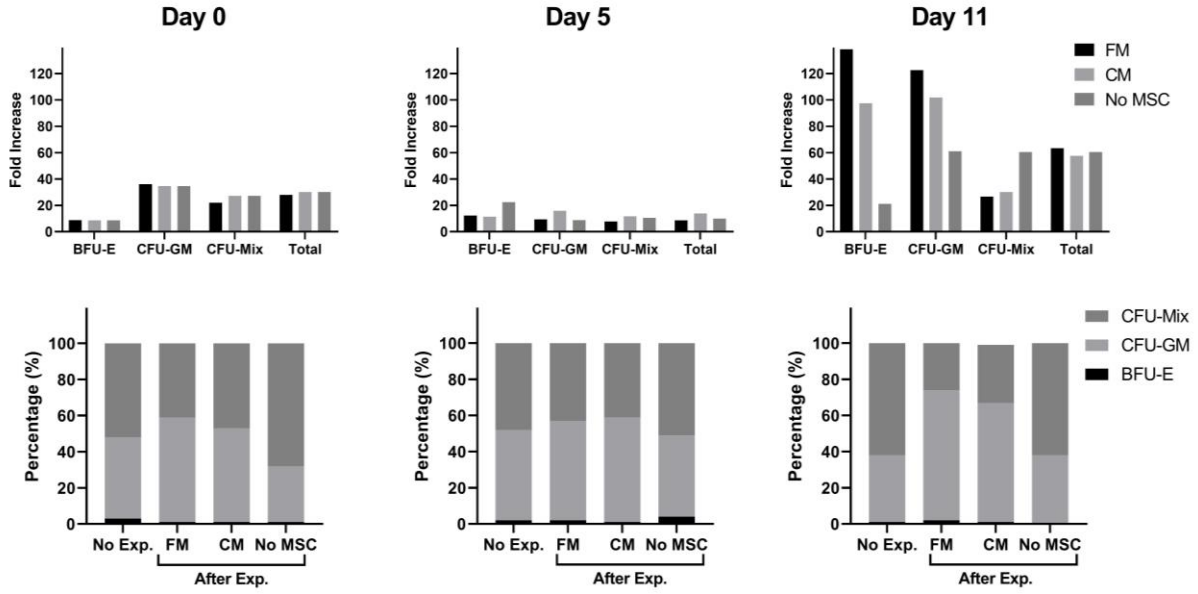

Figure 24 - Data regarding AT FBS. On top, fold increase in number of BFU-E, CFU-GM and CFU-Mix and total fold increase in the clonogenic potential of expanded HSPCs resulting from the hematopoietic support assays performed at days 0, 5 and 11 of the transport assays. At bottom, percentages of each colony type obtained before and after HSPC expansion, for each of the hematopoietic support assays.

4. Cost of Goods Analysis

4.1. Why is it important?

The great therapeutic potential of MSC-based therapies, namely how they may be able to answer long unmet clinical needs, was discussed in section 1.1.4. The development of off-the-shelf MSC-based products is thus very attractive. Nevertheless, as was seen in section 1.2, the manufacturing of MSCs is a complex and labor intensive process that still lacks optimization, involving strategic decisions regarding the appropriate technologies to use for the desired scale. Furthermore, the development and manufacturing of MSC-based products is extremely money consuming due to the high costs of the required GMP compliant facility and quality controls, as well as the expensive reagents and high labor costs[84]. Despite the fact that allogeneic cell therapies benefit from economies of scale, as the manufacturing process may be scaled up[191,192], the cost of goods (COG) remains elevated and may result in a final price that is prohibitive for broad adoption, causing reimbursement issues and eventual commercial failure. Proof of the importance of economic considerations in ATMPs development are products such as Glybera (Uniqure) and Chondrocelect (TiGenix), ATMPs approved by EMA and withdrawn from the market due to high prices, lack of reimbursement and limited number of patients, which resulted in an inviable business model[193].

COG analysis and optimization in the context of cell therapy products aims to minimize the cost per dose, while maintaining product quality[191]. Lipsitz and colleagues[191] developed a roadmap for cost of goods estimation in cell therapy products that recommends the consideration of "needle to needle" costs from the very beginning of product development, ensuring a viable product life cycle. COG analysis also allows for identification of main cost drivers, which may help to identify necessary process alterations in early developmental phases and highlight possible room for optimization, in an effort to ensure maximum efficiency and optimal resource allocation[125,194]. The goal of COG optimization is thus a more affordable product that will necessarily achieve more reimbursement and wider patient access[191,195].

Several studies can be found in the literature where bioprocess models and decisional tools are applied with the purpose of identifying the most cost effective technology for cell therapy manufacturing and/or verifying commercial feasibility and optimizing COGs[84,104,125,192,196–198]. In the context of this work, a COG analysis was performed with the goal of analyzing the costs of each production step and estimating cost per dose of an MSC-based product being developed by Stemlab SA. This serves as a benchmark that allows for more informed decisions regarding process changes in early stages of development and identifies the main cost drivers, which may be optimized.

4.2. Model Assumptions

The purpose of this section is to modulate the manufacturing process used by Stemlab SA in the production of an allogeneic MSC-based product in its early phases of development. Data regarding the manufacturing process employed by the company will be used to estimate all "needle-to-needle" production costs with the goal of determining the final cost per dose and identifying main cost drivers.

4.2.1. Manufacturing Process

The manufacturing process is divided into two phases, as can be seen in Figure 25. After the preliminary isolation and expansion steps, the obtained cells are processed and cryopreserved, in individual units, in the Master Cell Stock (MCS). When production is resumed, each unit from the MCS can be thawed and further expanded, being eventually subjected to a second cryopreservation step, this time on the Working Cell Stock (WCS). This separation into two production phases, delimited by the two cryopreservation steps allows for the implementation of an intermediate QC analysis before cells are added to the MCS, which will help to determine whether or not the process should move forward. Another advantage is that the initial cryopreservation step allows for a greater control of production timing as the units do not require to be processed at the same time and the work can be phased.

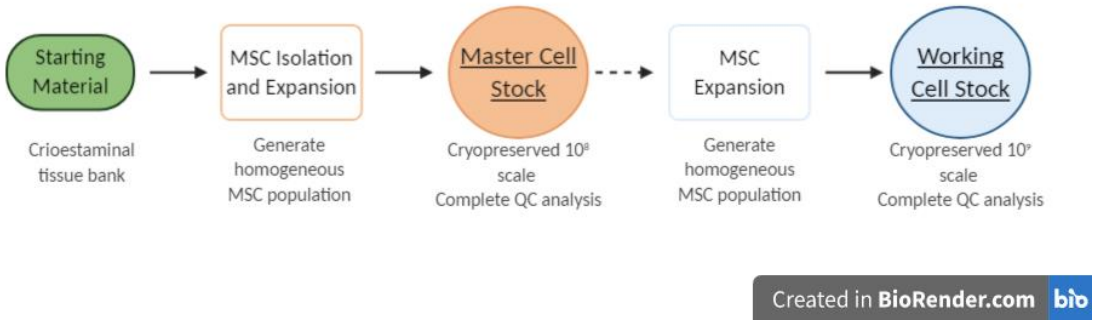

Figure 25 - Steps of the manufacturing process of an MSC-based product. Figure created with BioRender.com.

Due to the existence of two clear phases in this process that may be distant in time and require different resources, it makes sense to consider their COGs separately. Thus, the cost of one unit of the final product will correspond to the sum of the cost of one MCS unit and the cost of one WCS unit.

4.2.2. Production Scenarios

As was discussed previously, the number of *in vitro* passages cells are subjected to is an important variable with relevant weight not only on the quality of the final product but also on the COGs of the process. With this in mind, the three different bioprocessing scenarios detailed in Figure 26, varying in

the number of passages before each of the cryopreservation step, were analyzed and their COGs and number of cell population doublings were compared.

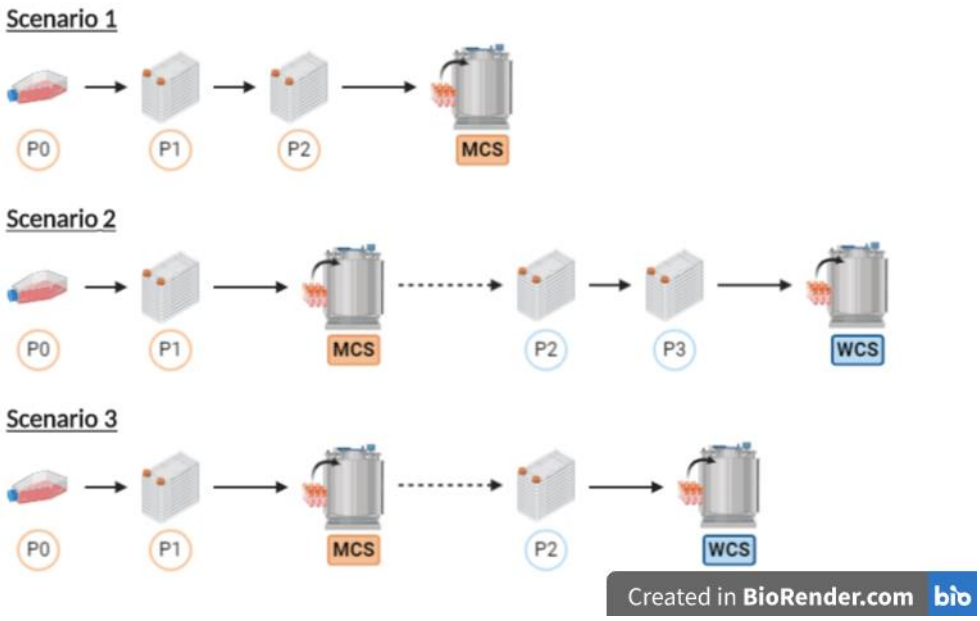

Figure 26 - Manufacturing scenarios considered. Figure created with BioRender.com.

4.2.3. Number of Cell Population Doublings

In each passage, cells are plated at a target density of 3 000 cells/cm^2 and passaged again when an estimated density of 75 000 cells/cm^2 is reached. By multiplying these values by the area of culture it is possible to obtain the number of cells seeded and the number of cells harvested, respectively, which, as was described in the Materials and Methods section, lets us obtain the NCPD.

4.2.4. Costs

For each production scenario, five different categories of costs were considered: reagents, materials, facilities, human resources and quality control. As was previously mentioned, the costs of the production of each cell stock were considered separately. For each of the cell stocks, the sum of all costs corresponds to the total cost of a batch.

4.2.4.1. Reagents

This cost category concerns all expenses with the commercially available bottled reagents necessary to perform the passaging and freezing steps in each production scenario. These include MEM alpha (22561021 Life Technologies), which is supplemented with 5% (v/v) HPL (HPCHXCRL50 Helio Bioscience) and 1% (v/v) Anti-Anti (15240062 Life Technologies), TrypLE (A1285901 Life Technologies) and PBS (A1285601 Life Technologies). For the estimation of reagents costs, the volume, in milliliters, of culture medium, TrypLE and PBS required in each passage was calculated based on the recommended working volume of each culture flask and the number of flasks used in each passage.

The volume of culture medium considered for each passage took into account the assumption that one medium change was performed between each passage.

The total necessary volume of each reagent was divided by the capacity of their respective commercially available packages and the result was rounded up, thus yielding the number of bottles used of each reagent. This number was multiplied by the respective cost per bottle and all costs were summed to obtain the total cost of reagents for one batch.

4.2.4.2. Materials

The main materials considered in estimation of materials expenses were T-175 (431306 Corning), Cell Stack 5 (734-4061 Corning) and Cell Stack 10 (734-1041 Corning), all used in MSC planar expansion. For each scenario, the number of necessary culture vessels was estimated based on the number of expected cells in each passage and the culture area required to continue expansion. This number was multiplied by their unitary cost and all costs were summed. To obtain the total material cost per batch, this result was increased in 10% to account for the small disposable materials commonly used in the lab such as pipette tips and falcon tubes.

4.2.4.3. Cryopreservation

Expenses with reagents and materials necessary for cryopreservation were considered to amount to 2% of the value of the total costs in each cell stock.

4.2.4.4. Facilities

Expenses with facilities correspond to the cost of occupying the GMP clean room during production. This room has a cost per day and per square meter of 3.93€, having a total area of 100m^2. Because the project only occupies 33m^2, the cost per day is 131.00€. Facility costs were estimated by multiplying this value by an estimation of the number of days required to produce each batch.

4.2.4.5. Human Resources

HR costs concern the wages of lab operators. This cost category was estimated based on the assumption that, for scenario 3, the HR costs per batch were 2072.00€ for the production of the MCS, which included two passages, and also 2072.00€ for the production of the WCS, which included one passage. For scenarios 1 and 2, which include an extra passage in the MCS and in the WCS respectively, the costs per batch were increased in 35% to account for the extra work necessary.

4.2.4.6. Quality Control

Costs with QC correspond to expenses with all testing performed before each cryopreservation step with the goal of ensuring product identity and quality. Because this step of the production is

outsourced the costs are fixed. The cost per batch of a battery of QC assays costs is 518.82€ for the MCS and 510.33€ for the WCS.

4.3. Model Results

The product produced in scenario 1 is subjected to three passages, representing a total of 12.5 population doublings, and only one cryopreservation step. Cells are thus cryopreserved in P2. As the WCS is eliminated, the final product is each unit of the MCS. A dose of the final product contains 100 million cells (based on the commonly applied dosage of 1 million cells per kg), so the number of doses that are possible to produce in this scenario is calculated by dividing the number of cells harvested in the last passage by 100 million, assuming that cryopreservation will represent loses of 25%. In scenario 1 it is thus possible to produce 14 doses and the final cost per dose is 907.17€.

In scenario 2, MSCs are passaged two times before cryopreservation in the MCS and another two times before cryopreservation in the WCS, which represents a total of 16.5 population doublings. In this scenario, the final product is each unit that composes the WCS, cryopreserved in P3, and the number of doses is obtained similarly to scenario 1. Unlike scenario 1, the number of units in the MCS is obtain by dividing the number of cells harvested in the last passage before the first cryopreservation by the number of cells required to start one WCS, considering, all the same, that the number of cells thawed is 25% less than the number of cells cryopreserved. This means that the total number of doses produced in scenario 2 is obtained by multiplying the number of doses in the MCS by the number of doses in the WCS. The MCS originates 37 doses and each of these doses is able to originate 14 doses in the WCS. The total number of doses produced in scenario 2 is thus 518 and the final cost per dose is 972.74€.

In scenario 3, the MCS is constructed similarly to scenario 2, yielding 37 doses. This scenario differs from scenario 2 in the number of passages before cryopreservation in the WCS, which in this case is only one, reducing the total NCPD to 13.5. Unlike scenario 2, the passage to expand cells for the WCS requires 4 units of the MCS and these are able to originate 7 doses on the WCS. The total number of doses produced in scenario 3 is thus 63 which corresponds to the product of 37 divided by 4 with the number of doses of the WCS. The cost per dose in this scenario is 1141.69€.

The main characteristics of each manufacturing scenario are summed up in Table 8. The lowest cost per dose in achieved in scenario 1, in which, unlike the other two scenarios, the final product is each unit of the MCS. In this scenario, by eliminating the second cryopreservation step, the manufacturing of the cell product must occur without interruptions, which can be logistically challenging. This manufacturing strategy may be useful and cost-effective if the desired goal is a product with just one cryopreservation step, although two freezing steps with at least one passage before freezing has been

shown to not compromise the final product's quality[152]. Considering this, scenarios 2 and 3 are both equally feasible. Scenario 2 has the second lowest cost per dose of the final product because of the increment on the number of doses caused by the increased NCPD (the highest of the three scenarios). As was seen previously, although most studies place the upper limit of NCPD on higher values, the best practice is to minimize the risks of cell ageing and senescence effects on the final product, which increase with passage number. Furthermore, as was discussed in the introduction, each passage, especially in 2D open culture platforms like the present case, increases the risks of contamination and introduction of batch to batch variability. Thus, it is important to carefully consider if the reduction in cost per dose obtained in scenario 2 is worth the extra passage and the increased NCPD. As in scenario 2, scenario 3 includes two freezing steps but only one passage before the second cryopreservation, which amounts to the same number of cell passages as in scenario 1. When compared with scenario 1, this scenario yields a considerable larger number of doses with only one extra population doubling, which may be advantageous. The cost per dose of scenario 3 is higher than that of scenario 2 as less doses are produced but the NCPD is kept considerably lower and below the limits indicated by the studies mentioned above. This means that scenario 3 may be a safer approach which will compensate for the more elevated cost per dose. Nevertheless, more variables must be considered when deciding which production scenario is the best choice, such as the logistical questions regarding the dimension of the GMP facility, the number of incubators available or the human resources necessary in each scenario, and the characteristics of the product being produced, namely the clinical target and the number of doses required.

Table 8 - COG analysis results for each production scenario.

		Scenario 1	**Scenario 2**		**Scenario 3**	
		MCS	MCS	WCS	MCS	WCS
Individual Cost Categories	Reagents	5 281.35€	2 158.46€	5 039.62€	2 158.46€	2 482.59€
	Materials	972.02€	318.73€	804.31€	318.73€	604.09€
	Facilities	2 882.00€	2 227.00€	1 441.00€	2 227.00€	786.00€
	HR	2 797.20€	2 072.00€	2 797.20€	2 072.00€	2 072.00€
	QC	518.82€	518.82€	510.33€	518.82€	510.33€
Total Cost	Cost/Batch	12 700.42€	7 440.91€	10 804.31€	7 440.91€	6 584.11€
	Units/Batch	14	37	14	37	7
	Cost/Unit	907.17€	201.11€	771.74€	201.11€	940.59€
	Cost/Dose	907.17€	972.84€		1 141.69€	
Final Product Features	No of Doses	14	518		63	
	Total No of Passages	3	4		3	
	Total NCPD	12.5	16.5		13.5	

5.4. Sensitivity Analysis

Following the estimation of COGs for each production scenario, a sensitivity analysis was performed with the goal of identifying the main cost drivers and, consequently, the best targets for eventual process optimization. This was done by analyzing the alterations, in percentage, to the final cost per dose caused by variations of 5%, 10%, 15%, 20%, 25% and 30% in each of the cost categories considered. The results obtained for production scenarios 1, 2 and 3 are represented in graphic form in Figure 27.

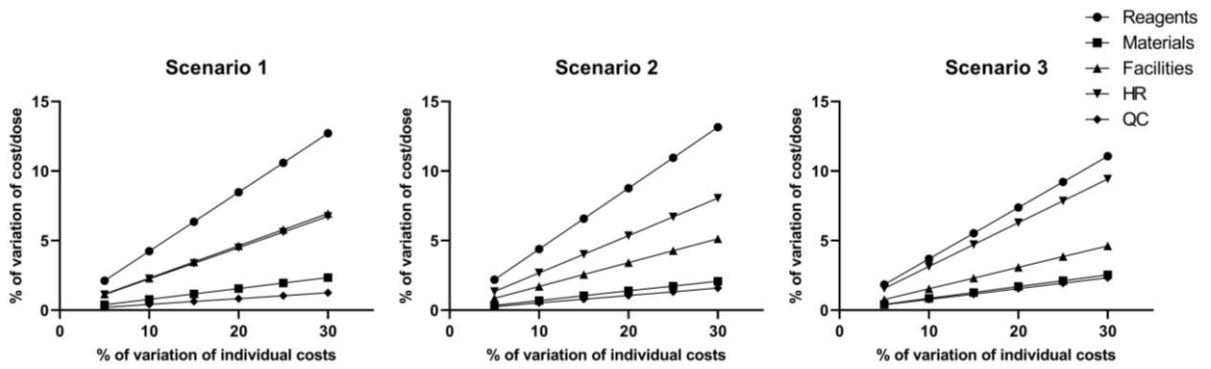

Figure 27 - Sensitivity of the cost/dose to variations in each individual costs in production scenarios 1, 2 and 3.

The results obtained for each scenario are relatively similar. In all figures it is possible to verify that the variations in Materials and QC costs have little impact on the final cost per dose, as the points corresponding to these two categories form the lines with the lowest slope, with changes of 30% causing a variation of around 2% in the total cost in all scenarios. On the other hand, it is clear that the final cost per dose is considerably more sensitive to Reagents costs, experiencing greater variations when this cost is increased or decreased, as is illustrated by the steeper slope of the line formed by these points in the three scenarios. Expenses with HR and Facilities appear in the middle of the graphs with varying slopes: in scenario 1 their influence on cost per dose is similar; in scenario 2, HR is a more relevant cost driver; and in scenario 3, cost per dose is more sensitive to Facilities expenses, its slope being close to that of Reagents. These observations are consistent with the weight of each cost category in the total manufacturing cost.

While the COG analysis serves as an indicator of how much will be spent in which individual cost category and predictor of the total costs, the sensitivity analysis lets us identify which of those cost categories are the best targets for optimization, seeing that the total cost has different sensitivities to different expenditure reductions, as was seen above. Ideally, to minimize cost per dose, the individual costs to which it is more sensitive must be minimized. The main cost drivers in the production of this MSC-based medicinal product are thus Reagents and, on a smaller degree, Facilities and HR. This means that efforts to implement process changes with cost optimization as a goal should be focused

mainly on these cost categories as these are the ones that will exert the biggest change in the final cost/dose.

6. Conclusions and Future Trends

MSCs are a controversial cell type: their mechanisms of action are not yet fully understood, nor is the response of the immune system when they are infused *in vivo*. Furthermore, their varying characteristics among different donors and sources raises the question of whether they constitute in fact one single cell population, with lack of consensus in the scientific community regarding their nomenclature. Regardless of this, the number of late-stage clinical trials and approved ATMPs using MSCs is fast increasing and the beneficial effect of these cells in the treatment of a myriad of pathologies is well established.

The manufacturing of MSC towards the development of cell therapies is a costly and logistically complex process that still requires optimization, especially for large-scale production. COG considerations should thus accompany technical decisions in every step of the manufacturing pipeline given that, as was seen in this work, even the number of cell passages and cryopreservation steps has a relevant influence on the final production cost and cost per dose of a cell product.

Regarding cryopreservation, which was previously established as a crucial step in the manufacturing process of an autologous, off-the-shelf MSC-based product, the detrimental effect that freeze-thaw cycles have on cells is widely accepted. Reviving strategies such as a short acclimation period in culture between cell thawing and infusion have been proven effective in circumventing these limitations. However, the adoption of such strategies in a production pipeline would increase the COG drastically, as expenditures with the use of more GMP facilities, more human resources and extra quality control would have to be accounted for. As this strategy is not yet economically feasible, the optimization of the cryopreservation and thawing procedures is a step towards the minimization of cell number losses, eventually leading to a reduction of cell dose due to improved function, which will positively reflect on the COG.

This work focused on this step of the manufacturing process with the goal of comparing different cryopreservation conditions. Results indicate that Cryostor CS5 represents a viable alternative to the standard (culture medium with 10% DMSO) and that cryopreservation up to two months does not have a particularly detrimental effect on the immunophenotype of MSCs. Future studies should focus on further cell characterization, namely plastic adherence and multilineage differentiation, and invest on assessing the effect of each cryopreservation solution on MSC function, particularly immunomodulation properties, crucial in the mechanism of action of MSCs against many clinical targets. It would also be of interest to re-plate MSCs post-thawing and re-evaluate them following an acclimation period in culture, in order to determine the degree of reversibility of the cryo-injury in each solution.

Transport solutions may prove to be useful in MSC manufacturing pipeline, either as an alternative to cryopreservation, capable of maintaining cell fitness in the case of short periods between manufacturing and patient infusion, or as a vehicle to transfer cells from the manufacturing site, where they are thawed, to the patient, without the need for further manipulation and quality control. This work demonstrated the efficacy of short-term hypothermic storage in 2-8 Cellsius for as long as 7 days and the ability of alginate encapsulation to maintain cell viability and function at RT for 11 days. Future work should assess the biocompatibility of these transport solutions, namely, the feasibility of infusing cells immediately following beads dissolution in comparison with, for example infusing cells post-thawing without removing DMSO. Additionally, possible regulatory issues regarding the inclusion of transport solutions in MSC production pipeline should be analyzed. Furthermore, similarly to cryopreservation solutions studies, it would be interesting to re-plate cells after transport and characterize them after some time in culture in order to investigate alterations.

In conclusion, the optimization of cryopreservation and short-term storage of MSC will decrease COG and increase the quality of the cells being produced, thus contributing to accelerate the clinical translation of these MSC-based products.

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