Integrated strategies for up- and down-stream processing of human mesenchymal stem cells

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Thesis to obtain the Master of Science Degree in Biological Engineering

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Abstract

Human mesenchymal stem cells (hMSC) are promising candidates for cell therapy applications. The main goal of this work was the development of a scalable integrated strategy for the up- and down-stream processing of hMSC. Different approaches for cell expansion, cell concentration (i.e. volume reduction) and washing steps were evaluated and the impact of operation mode on cells’ viability and quality (identity and potency) was compared.

For cell expansion, a xeno-free microcarrier culture system was successfully implemented in stirred tank bioreactors. A continuous perfusion operation mode allowed to increase the hMSC expansion ratio (13.4), and led to a metabolic shift towards an aerobic/oxidative metabolism, when compared to a semi-continuous operation mode. The perfusion culture was also validated using a cell retention perfusion device (ATF™).

For cell concentration and washing processes, tangential flow filtration (TFF) in hollow fiber devices was used. Cells were successfully concentrated up to a factor of 10, recovering 80% of viable cells. Membrane pore sizes larger than 0.45 µm have showed to be a key parameter to perform the cell concentration step. A continuous TFF allowed to operate longer with higher cell concentrations without affecting cell recovery yields during cell concentration step, and ensured higher protein clearance (98%) and lower cell death during cell washing than discontinuous TFF.

The best operating conditions obtained were selected for the development of an integrated bioprocess. After cell expansion in bioreactors operating in continuous perfusion, cells were harvested, concentrated and washed in a shorter total process time (when comparing with the total process time of separate processes), having been able to recover 70% of highly viable hMSC (> 95%). At the end of the bioprocess, hMSC displayed high cell viability and maintained their morphology, phenotype and multipotency.

Keywords: human Mesenchymal Stem Cells (hMSC); bioreactors; microcarriers; xeno-free; cell concentration; integrated process.
Resumo

As células estaminais mesenquimais humanas (hMSC) são candidatos promissores para aplicações em terapias celulares. O principal objectivo deste trabalho foi desenvolver uma estratégia integrada para a produção (upstream e downstream) de hMSC. Diferentes estratégias para expansão, concentração (redução de volume) e purificação das hMSC foram avaliadas, e o impacto de diferentes modos de operação na viabilidade e qualidade (identidade e potência) das células foi comparado.

Para a expansão de hMSC, um sistema de microcarriers num meio livre de componentes animais (xeno-free) foi implementado em bioreactores de tanque agitado. Operando em perfusão observou-se um aumento na expansão celular (13.4), e levou a um shift metabólico para um metabolismo aeróbio/oxidativo, quando comparando com um modo de operação semi-contínuo. O sistema de perfusão foi também validado usando um sistema de retenção de células (ATF™).

Os processos de concentração e diafiltração (lavagem) foram levados a cabo por via de filtração tangencial em módulos de fibras ocas. hMSC foram concentradas até 10 vezes, sendo possível recuperar 80% das células, com alta viabilidade. Membranas com diâmetros de poro superior a 0.45 µm mostraram ser um factor fulcral para o processo de concentração. Uma filtração operada em modo contínuo permitiu manter concentrações celulares mais altas durante o processo de concentração (sem afectar a recuperação de células) e permitiu remoções mais elevadas de proteína (98%) e níveis de morte celular mais baixos durante o processo de diafiltração.

As melhores condições de operação foram seleccionadas para o desenvolvimento de um bioprocesso integrado. Após expansão em reactores, hMSC foram recolhidas, concentradas e diafiltradas, obtendo-se tempos de processo totais mais baixos (quando comparando com vários processos em separado), e recuperando 70% de hMSC com alta viabilidade (> 95%). No final do processo, as hMSC conservaram as suas características, apresentando alta viabilidade e mantendo a sua morfologia, identidade e multipotência.

Palavras-chave: células estaminais mesenquimais humanas (hMSC); bioreactores; microcarriers; xeno-free; concentração de células humanas; processo integrado.
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List of Abbreviations

$\mu_{\text{max}}$  Maximum cell growth rate
2D  Two-dimensional
3D  Three-dimensional
ATP  Adenosine triphosphate
BM-hMSC  Bone marrow-derived human mesenchymal stem cell
BR  Bioreactor
BSA  Bovine serum albumin
DAPI  4’,6-diamidino-2-phenylindole
DMEM  Dulbecco’s modified Eagle medium
DMSO  Dimethyl sulfoxide
DO  Dissolved oxygen
DPBS  Dulbecco’s Phosphate-Buffered Saline
FACS  Fluorescence-activated cell sorting
FBS  Fetal bovine serum
FDA  Fluorescein diacetate
FSG  Fish skin gelatin
GLC  Glucose
GLN  Glutamine
GvHD  Graft versus Host disease
HF  Hollow fiber
hFF  Human foreskin fibroblast
hMSC  Human mesenchymal stem cell
HSA  Human serum albumin
IDO  Indoleamine-2,3-dioxygenase
IL  Interleukin
IMDM  Iscove’s Modified Dulbecco’s Medium
LAC  Lactate
LDH  Lactate dehydrogenase
MC  Microcarrier
MEM  Minimum Essential Media
NADH  Nicotinamide adenine dinucleotide
P/S  Penicillin-streptomycin
PDL  Population doubling level
PFA  Paraformaldehyde
PI  Propidium iodide
$q_{\text{net}}$  Specific consumption/production rate of a certain metabolite
TFF  Tangential flow filtration
<table>
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<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
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<tr>
<td>vvm</td>
<td>Volume of gas flow per volume of culture media per minute</td>
</tr>
<tr>
<td>$X_t$</td>
<td>Cellular concentration at the timepoint $t$</td>
</tr>
<tr>
<td>$Y_{LAC/GLC}$</td>
<td>Yield of lactate from glucose</td>
</tr>
<tr>
<td>$Y_{O2/GLC}$</td>
<td>Ratio of oxygen consumed per glucose</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Background

Human mesenchymal stem cells (hMSC), due to their characteristics, have been attracting attention from researchers in basic science and in the clinical field for the past years (Figure 1.1); their particular immunomodulatory characteristics [1], as well as their capacity in secreting bioactive molecules with anti-inflammatory and regenerative features [2], make them attractive candidates for autologous and allogeneic therapies.

![Figure 1.1 – Number of published papers per year related with the keyword Mesenchymal stem cells. Data obtained from Pubmed database.](image)

Today, more than 350 clinical trials are being conducted, mainly in Europe, China and USA (Figure 1.2). Furthermore, various completed trials already demonstrated the safety and efficacy of hMSC for therapeutical use in several diseases [3].

The ongoing/concluded clinical trials using hMSC focus on several disorders. Mostly, these are trials in Phase I (safety studies) or Phase II (proof of concept for efficacy in human patients) studies. In the following section are specified some concrete clinical studies using hMSC for the treatment of disorders such as graft-versus-host disease (GVHD), bone, cartilage and cardiovascular diseases.
1.1.1 Clinical applications of hMSC

In this section some of the current clinical trials are more thoroughly described. Figure 1.3 shows the distribution of the current clinical trials with hMSC according to the disease type being studied.

![Map showing distribution of clinical trials](image)

Figure 1.2 – Ongoing or completed clinical trials using hMSC. Data obtained from the clinicaltrials.gov public database on 10.10.2014.

![Pie chart showing disease distribution](image)

Figure 1.3 – Clinical trials of hMSC classified by disease type. Data obtained from the clinicaltrials.gov public database at December, 2011. Adapted from [3]
Graft-versus-host disease (GVHD): GVHD occurs after allogeneic hematopoietic stem cell transplant. This disorder is currently treated with corticosteroids, a treatment only showed to be effective for some patients [4]. The studies of Le Blanc, K. et al were the first to report successful results on the usage of hMSC in a subject which was treatment-resistant to acute GVHD of the gut and liver [5]. The treatment showed striking immunosuppressive effects, and the patient was reported to be healthy after 1 year. This group reports that from their experience of 1000 allogeneic stem cell transplantations, 25 patients developed acute GVHD (2.5%), and from those patients this one was the only patient who survived. This was therefore a study which encouraged the study of hMSC for the treatment of GVHD. Ringdén, O. et al reported in 2006 the successful treatment of 6 (out of 8) patients [6]. Following these studies, others confirmed the beneficial effect of hMSC on the treatment of this disease, posing hMSC as a potential novel therapy for GVHD [7 – 14]. Cell dosage used in these studies was in the range of 1 to 8 million cells per kg of the patient body weight. Also for the treatment of GVHD, Prochymal®, a stem cell therapy product from Osiris Therapeutics (Columbia, Maryland), is the first stem cell drug therapy approved in Canada. Although results have not been published, Osiris Therapeutics already completed Phase 3 clinical trials with Prochymal®, infused at 2 million hMSC per kilogram of the patient, in combination with corticosteroids (data obtained from clinicaltrials.gov database).

Bone and cartilage diseases: Osteogenesis imperfecta (OI) is a disease characterized by skeletal fragility and connective tissue alterations caused by alteration of type I collagen production by osteoblasts. Pediatric patients with OI underwent allogeneic hematopoietic stem cell transplantation and the transplanted bone marrow cells engrafted and generated functional osteoblasts leading to improvement in bone structure and function [15]. It is important to refer that this study was performed with whole bone marrow and not isolated hMSC. In a follow-up study from the same group, patients were infused with allogeneic transplantation of purified hMSC, showing further benefit in relation to whole bone marrow transfusions [16]. Regarding cartilage diseases, studies were already performed on joint defects. Wakitani S. et al, transplanted autologous culture-expanded bone marrow mesenchymal cells (embedded in a collagen gel) into nine full-thickness articular cartilage defects of the patello-femoral joints, with significant improvements on cartilage repair [17]. This group, using the same technique (hMSC embedded in collagen gel), treated patients with knee osteoarthritis being shown reduced recovery times [18].

Cardiovascular diseases: Due to their high replicability, paracrine effect, ability to preserve potency, and lack of adverse reactions to allogeneic transplants [119-120], hMSC became an attractive candidate for cardiovascular repair in cases of ischemic heart disease and congestive heart failure (major causes of morbidity and mortality). Preliminary studies in animal models in 2006 showed an improved repair of infarcted myocardium after hMSC administration [19]. Tests were also performed on humans after acute myocardial infarction. It was seen a significant increase on left ventricular function after intracoronary injections of autologous bone marrow hMSC [20]. The company Baxter (Illinois, USA) is currently performing Phase 3 clinical trials with autologous bone marrow-derived CD34+ endothelial progenitor cells for the treatment of chronic myocardial ischemia [89].
1.1.2 hMSC characterization

Mesenchymal stem cells were firstly discovered in 1968 by Friedenstein [88] and since then a consensus about the real definition of hMSC was never thoroughly uniformed. To overcome problems of this origin and to better define what a hMSC is, in 2006, the International Society of Cellular Therapy established three minimal criteria to define hMSC (Table 1.1) [21].

Table 1.1 - Minimal criteria for defining multipotent mesenchymal stromal cells. [21]

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
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<tbody>
<tr>
<td>Adhesion capacity</td>
<td>hMSC must be adherent to plastic under standard tissue culture conditions</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Positive (&gt; 95%) CD73 CD90 CD106</td>
</tr>
<tr>
<td></td>
<td>Negative (&lt; 2%) CD34 CD45 CD14 or CD11b CD79α or CD19 HLA-DR</td>
</tr>
<tr>
<td>Multipotency</td>
<td>hMSC must have the capacity to differentiate into osteoblasts, adipocytes</td>
</tr>
<tr>
<td></td>
<td>and chondroblasts under in vitro conditions</td>
</tr>
</tbody>
</table>

1.1.2.1 hMSC source

In 1976, Friedenstein [22] was able to isolate clonogenic fibroblast precursor cells (CFU-F) from the bone marrow (BM) of mice. BM was therefore taken as the main hMSC source during several years. However, isolating hMSC from BM is a highly invasive procedure and the number, differentiation potential, and maximum life span of cells decline with increasing age of the donor [23]. To put in perspective, the ratio of hMSC per number of BM cells is of 1:10.000 in a newborn child, while in a 30 year old adult this ratio is of 1:250.000 [24], which represents an occurrence 25 times smaller. Therefore, other sources for hMSC such as umbilical cord blood (UCB) or adipose tissue (AT) were introduced as potential alternatives (Figure 1.4). These sources pose their own advantages and disadvantages for their clinical use (summarized in Table 1.2).

<table>
<thead>
<tr>
<th>Adult tissues</th>
<th>Fetal/neo-natal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Bone marrow</td>
<td>• Amniotic fluid</td>
</tr>
<tr>
<td>• Peripheral blood</td>
<td>• Human amnion membrane</td>
</tr>
<tr>
<td>• Adipose tissue</td>
<td>• Chorion membrane</td>
</tr>
<tr>
<td></td>
<td>• Chorion villi</td>
</tr>
<tr>
<td></td>
<td>• Decidua</td>
</tr>
<tr>
<td></td>
<td>• Placenta</td>
</tr>
<tr>
<td></td>
<td>• Cord blood</td>
</tr>
<tr>
<td></td>
<td>• Wharton’s jelly</td>
</tr>
<tr>
<td></td>
<td>• Umbilical cord</td>
</tr>
</tbody>
</table>

**Figure 1.4 – Available sources for hMSC.** The sources can be distinguished between adult and neo-natal birth-associated tissues [25].
Since cell number and invasive harvest procedures are two of the main problems in obtaining hMSC, a non-invasive source that could provide high hMSC number would be ideal. Adipose tissue is a source which is widely available from liposuction procedures but, as reviewed in other studies, the amount of hMSC in the tissues decreases with age, posing a problem not of tissue amount, but of hMSC occurrence in that tissue. However, the BM remains the most studied source of hMSC, being therefore still the most used.

**Table 1.2 – Advantages and disadvantages of several hMSC sources.** (BM: bone marrow; AT: adipose tissue; UCB: umbilical cord blood)

<table>
<thead>
<tr>
<th>MSC source</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| BM         | • Main source (most studied)  
• High isolation efficiency  
• Proliferative | • Highly invasive  
• Number and quality decline with donor’s age | [25, 27-28] |
| AT         | • Less invasive  
• Large quantities  
• High isolation efficiency  
• Proliferative | • Number and quality decline with donor’s age | [25-28] |
| UCB        | • Less invasive  
• Proliferative | • Moral issues | [25, 28] |

### 1.1.2.2 Therapeutical characteristics of MSC

As mentioned before, hMSC have biological characteristics which contribute for their therapeutical effects. The most important MSC characteristics are schematized in Figure 1.5, and are further developed in this section.

**Capacity to migrate and engraft:** hMSC have the ability to home to sites of inflammation following tissue injury when injected intravenously. This capacity is dependent on several signals (*e.g.* growth factors and cytokines), which can be secreted either by the injured cells or by the immune cells responding to the injury. Growth factors such as platelet-derived growth factor (PDGF) or insulin-like growth factor 1 (IGF-1) and cytokines such as CCR2, CCR3, CCR4 or CCL5 have been proven to control hMSC migration [30, 31]. This capacity was already observed for several pathologic conditions, irrespectively of the tissue. MSC were shown to home to lung in response to injury, adopt an epithelium-like phenotype and reduce inflammation in mice affected with bleomycin [29]. Also in mice, MSC were able to migrate to injured muscle tissue [32].
**Multipotency:** hMSC have the capacity to differentiate (both *in vivo* and *in vitro*) into mesenchymal lineages such as adipocytes, chondroblasts and osteoblasts. Nonetheless, other studies have shown that hMSC can differentiate into other cell lineages such as lung cells [29, 33-35], kidney cells [38], astrocytes and neurons [36, 37]. In a specific case study, using mice with an ischemic kidney disorder, the transplanted MSC were able to differentiate towards renal tubular epithelium. The differentiated donor cells replaced the vacant space left over by the dead cells, therefore contributing to the maintenance of structural integrity and preceded to a subsequent tissue repair process [38].

**Secretion of bioactive molecules:** hMSC have the ability to secrete several bioactive molecules, such as growth factors or cytokines, which have modulatory effects on the tissue microenvironment, from anti-inflammatory to anti-apoptotic or even regeneration-stimulating. The mode of action can be direct, through intracellular signaling or indirect, through stimulation of other cells to secrete certain bioactive molecules as well. A study from Takahashi et al demonstrated that specific cytokines secreted by hMSC contributed to functional improvement of the infarcted heart by inhibiting apoptosis and inducing therapeutic angiogenesis [39]. The effect of these bioactive molecules is so remarkable that studies have shown positive effects in cases of liver failure, just by using the conditioned medium from hMSC [40]. Some examples of the molecules secreted by hMSC and their specific functions are indicated in Table 1.3.
Table 1.3 – Important bioactive molecules secreted by hMSC and their function.

<table>
<thead>
<tr>
<th>Bioactive molecule</th>
<th>Function</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Interleukin-10 (IL-10)</td>
<td>anti-inflammatory</td>
<td>[41, 42]</td>
</tr>
<tr>
<td>Transforming growth factorβ-1 (TGFβ1), hepatocyte growth factor (HGF)</td>
<td>suppress T-lymphocyte proliferation</td>
<td>[43]</td>
</tr>
<tr>
<td>Prostaglandin-E2 (PGE2)</td>
<td>anti-proliferative mediator, anti-inflammatory</td>
<td>[44, 45]</td>
</tr>
<tr>
<td>Endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PIGF), monocyte chemoattractant protein-1 (MCP-1)</td>
<td>enhance proliferation of endothelial cells and smooth muscle cells</td>
<td>[46, 47]</td>
</tr>
<tr>
<td>Interleukin-1 receptor antagonist</td>
<td>anti-inflammatory</td>
<td>[48]</td>
</tr>
<tr>
<td>LL-37</td>
<td>anti-microbial peptide and anti-inflammatory</td>
<td>[49]</td>
</tr>
<tr>
<td>Human leukocyte antigen G isoform (HLA-G5)</td>
<td>anti-proliferative for naïve T-cells</td>
<td>[50]</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>restore epithelial protein permeability</td>
<td>[51]</td>
</tr>
<tr>
<td>Keratinocyte growth factor</td>
<td>alveolar epithelial fluid transport</td>
<td>[52]</td>
</tr>
<tr>
<td>MMP3, MMP9</td>
<td>mediating neovascularization</td>
<td>[53]</td>
</tr>
</tbody>
</table>

**Immunomodulatory function:** hMSC are known to modulate the immune system, having unique characteristics that allow their persistence in a xenogeneic environment. Even though the precise mechanisms regulating this capacity are not fully understood, the scientific community hypothesizes that cell-cell contact or the bioactive molecules secreted by hMSC (referred on the last point) could be playing major roles [3]. This is a characteristic which raised a great amount of interest in the potential of hMSC in the treatment of immune disorders such as GVHD. Table 1.4 contains a short summary on the effects of hMSC in some immune cells [89-91].
Table 1.4 – Immunomodulatory effects of hMSC.

<table>
<thead>
<tr>
<th>Immune cell</th>
<th>hMSC effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendritic cells (DC)</td>
<td>Influence on differentiation, maturation and function of DC; suppress DC migration and antigen presentation; induce mature DC into a novel Jagged-2-dependent regulatory DC population.</td>
<td>[54-56]</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Alter the cytokine secretion profile of T cells; suppress T cell proliferation; promote the expansion and function of Treg cells.</td>
<td>[43, 57-58]</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>Inhibit B cell proliferation; affect B cell chemotactic properties; suppress B cell terminal differentiation.</td>
<td>[59-61]</td>
</tr>
<tr>
<td>NK cells</td>
<td>Alter NK cells phenotype and suppress their proliferation, cytokine secretion and cyto-toxicity.</td>
<td>[62, 63]</td>
</tr>
</tbody>
</table>

1.2 Bioprocessing of hMSC for clinical applications

Clinical trials from the early stages have so far indicated that the use of both autologous and allogeneic hMSC appears to be safe [5-8]; however, efficacy has not been demonstrated in recent late-stage clinical trials [64]. Once the tissue source to obtain hMSC is determined for an intended clinical application, the safety and efficacy of cell therapeutics produced may be significantly influenced by cell bioprocessing. As a consequence, developing robust expansion processes by optimizing culture variables is critical to efficiently and consistently generate hMSC that retain desired regenerative and differentiation properties while minimizing any potential risks [65, 66].

![Figure 1.6 – Advantages and disadvantages provided by planar and microcarrier-based stirred culture systems.](image-url)
Due to hMSC low frequency in body tissues and in order to guarantee the cell numbers required for therapy (1 to 2 million of hMSC per kilogram body weight of the patient is generally suggested [3]), hMSC expansion in vitro is imperative. Traditionally, hMSC are cultured in 2D planar systems (e.g. Petri dishes, culture flasks and well plates). The inherent uncontrollability, heterogeneity and low production yields associated with these planar technologies have made them unattractive and unsuitable for clinical and industrial applications. Therefore, microcarrier-based culture systems in stirred bioreactors have been, up to the date, an attractive alternative system for hMSC expansion. In Figure 1.6 is presented a summary on the advantages of planar and microcarrier-based stirred culture systems, as well as the challenges each system faces.

Similarly, downstream processes face their own challenges, and will have to follow closely the advances being made in the upstream field. As the new upstream technologies continue to increase the productivity of cell cultures for therapeutical applications, cell yields are expected to increase exponentially per lot [68]. With cell harvest volumes expected to reach up to 1000 L over the following 10 years, the challenges appear in the following steps of the process: cell harvest and purification (including efficient cell-microcarrier separation), cell concentration (volume reduction), cell washing, formulation, freezing and storing. These challenges and the respective solutions that downstream processes will need to provide are summarized in Table 1.5.

<table>
<thead>
<tr>
<th>Product requirement at the clinic after thaw</th>
<th>Relation to DSP requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;80% viability</td>
<td>Frozen at &gt;85% viability;</td>
</tr>
<tr>
<td></td>
<td>Well controlled freezing profiles;</td>
</tr>
<tr>
<td></td>
<td>Controlled processing times, &lt;4 h per unit operation;</td>
</tr>
<tr>
<td></td>
<td>Same-day processing.</td>
</tr>
<tr>
<td>Specified number of viable cells</td>
<td>Consistent and accurate fill volumes;</td>
</tr>
<tr>
<td>&gt;80% viable cell recovery</td>
<td>Minimal volume loss during thaw;</td>
</tr>
<tr>
<td></td>
<td>Robust formulation step.</td>
</tr>
<tr>
<td>10 – 100 million cell/mL</td>
<td>Achieve high concentration factor during volume reduction step with minimal cell loss or damage;</td>
</tr>
<tr>
<td></td>
<td>Low system volume (hold-up volume) in the volume reduction step.</td>
</tr>
<tr>
<td>Single-cell suspension</td>
<td>No/minimal clumps of cells at fill;</td>
</tr>
<tr>
<td></td>
<td>No pelleting.</td>
</tr>
<tr>
<td>Impurities &lt;1 ppm</td>
<td>High efficiency wash step;</td>
</tr>
<tr>
<td></td>
<td>Cells in suspension during wash step;</td>
</tr>
<tr>
<td></td>
<td>Low system volume.</td>
</tr>
<tr>
<td>Maintenance of product quality attributes</td>
<td>No cell damage;</td>
</tr>
<tr>
<td></td>
<td>Low-shear processing.</td>
</tr>
</tbody>
</table>
1.2.1 Ex-vivo expansion of hMSC

As stated before, moving hMSC cultures from planar, static systems to microcarrier-based, stirred systems is imperative to enhance cells’ performance and fully exploit cells’ potential. By providing a cellular context closer to what actually occurs in native microenvironment, these strategies can significantly improve cells’ viability, identity and function [97-99].

Lack of homogeneity in the culture medium is a considerable drawback of planar culture systems, while in stirred systems is provided a more homogeneous environment to the cell culture, in terms of nutrient delivery, oxygen transfer rates and temperature. Agitation is essential for ensuring that all cells are exposed to culture medium of the same composition while keeping the microcarriers suspended [87]. The lack of monitoring of culture variables is another disadvantage of planar systems, which can be overcome in a bioreactor through the use of electrodes and by the ability of non-destructive sampling.

Due to the fact that hMSC are adherent cells, the availability of surface area becomes a critical factor for these cultures. Microcarrier technology is a solution that provides high surface areas in small volumes, being to the date the most commonly used technique to grow hMSC in stirred suspension.
culture systems. This opens up even more the possibility of scaling up hMSC culture systems. Increasing the scale of bioreactor cultures would pose some challenges, such as the increase in shear stress due to the need of bigger stirrers or higher stirring speeds, in order to maintain the homogeneity in a larger volume [121]. Nevertheless, stirred tank bioreactors are still the most used strategy for hMSC cultures. Some examples of the most used bioreactor configurations in stem cell bioprocessing are presented in Figure 1.7. Table 1.6 includes a summary of some studies already performed with hMSC in several bioreactor systems.

Table 1.6 – Summary of the studies involving the cultivation of hMSC in different bioreactor systems.
(MC: microcarriers; DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; FCS: fetal calf serum; EMEM: Eagles Minimal Essential Medium; ST: stirred tank; n.d.: not defined)

<table>
<thead>
<tr>
<th>Culture Strategy and Conditions</th>
<th>Results (Fold increase in cell concentration; X\text{max})</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>System: Spinner flask</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume: 80 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium: StemPro® hMSC SFM XenoFree</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy:</td>
<td></td>
<td>[100]</td>
</tr>
<tr>
<td>BM-hMSC in Cultispher-S MC</td>
<td>18-fold in 14 days; X\text{max}: 2x10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>AT-hMSC in Cultispher-S MC</td>
<td>14-fold in 14 days; X\text{max}: 1.4x10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>System: Spinner flask</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume: 100 mL</td>
<td></td>
<td>[91]</td>
</tr>
<tr>
<td>Medium: low glucose DMEM, 20% FBS and supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: BM-hMSC in Cytodex-3 MC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System: Mobius CellReady® (disposable)</td>
<td>X\text{max}: 2x10^5 cell/mL</td>
<td>[101]</td>
</tr>
<tr>
<td>Volume: 3 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium: DMEM and 10% FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: BM-hMSC in MC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System: Fixed-bed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume: 4x14 mL</td>
<td>285-fold in 5.5 days; X\text{max}: 2.9x10^6 cell/mL</td>
<td>[102]</td>
</tr>
<tr>
<td>60 mL</td>
<td>26-fold in 7 days; X\text{max}: 1.8x10^6 cell/mL</td>
<td></td>
</tr>
<tr>
<td>300 mL</td>
<td>18-fold in 7 days; X\text{max}: 2.1x10^6 cell/mL</td>
<td></td>
</tr>
<tr>
<td>Medium: EMEM and 10% FCS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: hMSC-TERT in non-porous borosilicate glass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System: Stirred-tank (ST) bioreactor</td>
<td></td>
<td>[95]</td>
</tr>
<tr>
<td>Volume: 800 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium: StemPro® hMSC SFM XenoFree</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM-hMSC in Plastic MC</td>
<td>~6.5-fold in 7 days; X\text{max}: 1.3x10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>AT-hMSC in Plastic MC</td>
<td>~3-fold in 7 days; X\text{max}: 5.7x10^4 cell/mL</td>
<td></td>
</tr>
<tr>
<td>BM-hMSC in Plastic MC (Perfusion in 400mL ST)</td>
<td>~17-fold in 11 days; X\text{max}: 5.0x10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>System: Hollow fiber fibronectin-coated bioreactor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume: n.d.</td>
<td>20-fold in 13 days; X\text{harvest}: 98x10^6 cells</td>
<td>[103]</td>
</tr>
<tr>
<td>Medium: low glucose DMEM and 10% human platelet lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: BM-hMSC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System: Stirred-tank (ST) bioreactor</td>
<td></td>
<td>[104]</td>
</tr>
<tr>
<td>Volume: 2.5 L</td>
<td>6-fold in 9 days; X\text{max}: 1.6x10^6 cell/mL</td>
<td></td>
</tr>
<tr>
<td>Medium: DMEM, 10% FBS and supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: BM-hMSC in Plastic MC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System: Stirred-tank (ST) bioreactor</td>
<td></td>
<td>[105]</td>
</tr>
<tr>
<td>Volume: 1.5 L</td>
<td>10-fold in 8 days; X\text{max}: 2.6x10^6 cell/mL</td>
<td></td>
</tr>
<tr>
<td>Medium: DMEM/F12, 3% FBS and supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strategy: BM-hMSC in Cytodex-3 MC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.1.1 Microcarriers

As stated before, microcarrier technology has the capacity of increasing greatly the ratio of surface area per unit of culture volume. This has positive implications on the scalability of the process, as well as in process costs (regarding culture media, for example). Economic analysis of bioprocesses has already demonstrated that above certain production scales, planar technologies will cease to be cost-effective and microcarrier-based bioreactors will become the only option [129]. Microcarrier cultures also offer the possibility of increasing the number of the culture passages by the addition of empty microcarriers during culture time. This strategy allows for hMSC to transfer from bead to bead, colonizing the empty microcarriers that were added, extending culture periods without the need for an enzymatic dissociation [67, 96].

A variety of microcarriers for hMSC culture is commercially available today. Each product can be different in terms of its composition, charge or porosity. Some of the most common commercial microcarriers and their characteristics are presented in Table 1.7. Microporous microcarriers have pores with diameters smaller than 1 µm, while macroporous ones have pores with diameters usually above 10 µm. Furthermore, these microcarriers can be functionalized with specific coating materials (e.g. ECM proteins or small molecules) according to the culture needs, usually developed/sold by the same manufacturers.

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Porosity</th>
<th>Composition</th>
<th>Surface area/mass (cm²/g)</th>
<th>Manufacturer</th>
<th>Studies with hMSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytodex 1</td>
<td>Microporous</td>
<td>Dextran</td>
<td>4400</td>
<td>GE Healthcare</td>
<td>[67, 90, 94]</td>
</tr>
<tr>
<td>Cytodex 3</td>
<td>Microporous</td>
<td>Dextran</td>
<td>2700</td>
<td>GE Healthcare</td>
<td>[90, 91, 94]</td>
</tr>
<tr>
<td>Cultispher S</td>
<td>Macroporous</td>
<td>Gelatin</td>
<td>NA</td>
<td>Percell</td>
<td>[92, 93, 94]</td>
</tr>
<tr>
<td>Hillex</td>
<td>Microporous</td>
<td>Polystyrene</td>
<td>515</td>
<td>SoloHill Engineering</td>
<td>[94]</td>
</tr>
<tr>
<td>Plastic</td>
<td>Non-porous</td>
<td>Polystyrene</td>
<td>480</td>
<td>SoloHill Engineering</td>
<td>[95]</td>
</tr>
<tr>
<td>Synthemax II</td>
<td>Non-porous</td>
<td>Polystyrene</td>
<td>360</td>
<td>Corning</td>
<td>[96]</td>
</tr>
</tbody>
</table>

1.2.1.2 Operation modes

It has been shown before that the feeding regimen is a key factor for successful cell growth and proliferation in culture [108]. Batch cultures, typically used in the biopharmaceutical industry, have the drawbacks of nutrient depletion (such as glucose and glutamine) and by-products accumulation (such as lactate and ammonia) [122]. To avoid these limitations and to improve cell metabolism and growth, fed-batch and perfusion operation modes started being explored as alternative strategies to standard media exchange approach (so called semi-continuous). In animal cell cultures, fed-batch strategies with the feeding of nutrient concentrates has proven to be successful, operationally simple, reliable and flexible [123-124]. Continuous perfusion systems, with constant addition of fresh media and removal of exhausted media, may be more challenging to set up and operate (specially at larger scales).
scales) [125], but they allow for the maintenance of low concentration set points of the major carbon substrates, enabling a more efficient primary metabolism and leading to lower rates of production of metabolites such as ammonia and lactate [122].

In a spinner flask bioreactor (100 mL), dos Santos et al observed that a medium exchange of 25% every 2 days was not enough, and resulted in glucose starvation and in ammonia accumulation at potentially inhibitory concentrations [108]. This was solved initially by performing additional medium exchanges: 25% everyday, starting at day 3. With this strategy, glucose and glutamine concentrations were maintained at higher values and lactate and ammonia concentrations were maintained below inhibitory levels [108]. A perfusion strategy was later implemented in stirred tank bioreactors (400 mL), operating at a dilution rate of 0.25 day\(^{-1}\). Also in this case, nutrients were kept below depletion and products were maintained below inhibitory levels [95].

Animal cells growing in suspension in perfusion systems require a cell/microcarrier retention device in order to retain all cells inside the bioreactor while removing exhausted media [126, 128]. However, in microcarrier-based systems, a cell retention device is not essential most times, since microcarriers usually sediment at higher speeds than the linear speed at which the exhausted media is removed [127]. Nevertheless, cell retention systems still pose a great advantage in terms of downstream processing. In a scenario where cells are detached from the microcarriers still inside the stirred tank bioreactor, a retention device with a pore size smaller than the microcarriers size and bigger than the cells diameter, would allow for separation of microcarriers from the cell suspension without the need of a second process that would perform this separation. This integration of processes is already available with the Alternating Tangential Flow (ATF™, Repligen) technology. Even though scaling up and sterility can be recurring problems for cell retention systems, the possibility of process integration is very appealing [85].

### 1.2.2 Downstream processing of hMSC

The requirements for downstream processing (DSP) steps will be dictated by the requirements of the final product at the clinic, as described above (Table 1.5).

When a cell therapy product dose is thawed, it is expected to have a minimum viability for transplantation purposes. To achieve the required values, cell viability must be monitored and controlled through each step of the DSP. Low-shear cell concentration (i.e. volume reduction) and washing steps also need to be designed, as well as freezing operations with uniform and controlled-rate freezing profiles. Controlling processing times for DSP steps and keeping them to a minimum is also important to ensure that the processed cells can be cryopreserved on the same day they were harvested.

As DSP technologies are scaled up, disposable process analytical technologies will enable real time data collection and decision making so that processes can be continuously monitored and product quality can be controlled. Bench-top systems are able to process culture volumes up to 10 L. However, when those culture volumes increase, closed and scalable technologies such as tangential flow filtration (TFF) or continuous flow centrifugation are likely to be required [69, 70]. Figure 1.8 illustrates a typical workflow for a cell therapy manufacturing process, in this case for the blood-
processing industry. Each unit operation has unique process requirements that depend on total harvest yields, target cell dose and concentration, and total product doses per lot.

Figure 1.8 – Process-flow diagram of a typical cell therapy DSP – blood processing [69].

1.2.2.1 Available DSP technology

As volumes and lot sizes increase, the traditional centrifuge would require multiple rounds of centrifugation with several operators, becoming time consuming and cost prohibitive. Multiple technologies adapting, centrifugation, separation and filtration principles from the blood processing field have emerged to accommodate the cell therapy industry volume reduction and washing steps [69]. The inevitable need is for new scalable DSP technologies to prevent future bottlenecks in large-scale manufacturing of cell therapy products. These bioprocess technologies can be separated into filtration, size-exclusion and continuous/counterflow centrifugation. Table 1.7 presents the details of some of these technologies which have the potential for large-scale applications in DSP of cell therapy products.

Tangential flow filtration (TFF): TFF is a well-established filtration-based technology, which directly competes with centrifugation, depth filtration, and expanded bed chromatography for the initial clarification of therapeutic proteins from mammalian, yeast, and bacterial cell cultures [71, 72]. In a TFF, fluid is pumped tangentially along the surface of a membrane while pressure across the filter – transmembranar pressure (TMP) – drives it through the membrane to the filtrate side. It has also been commonly used for the purification and concentration of virus particles [73]. Because this technology is widely used in these fields, there is an increased offer of fully automated, disposable and integrated
(concentration and washing) TFF systems, with a wide range of processing capacities due to the variety of filter areas available. Therefore, process development costs can be reduced due to the lower cell numbers required for a meaningful run, unlike what may occur when using continuous-centrifugation systems. Beside the flexibility, TFF also presents other advantages such as a relatively linear scale-up behavior and fairly low shear forces and pressures (as microfiltration) applied throughout the process [74, 75].

Table 1.8 – Comparison between some commercially available technologies for the DSP of cell therapy products [69]

<table>
<thead>
<tr>
<th>Technology</th>
<th>CTI-TFF</th>
<th>TFF-Bioprocessing</th>
<th>Fluidized Bed</th>
<th>Conventional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Lonza</td>
<td>GE Healthcare</td>
<td>KBI Biopharma</td>
<td>Carr Centritech</td>
</tr>
<tr>
<td>Product name</td>
<td>CT-TFF</td>
<td>Uniflex</td>
<td>kSep 400 and 6000S</td>
<td>Unifuge</td>
</tr>
<tr>
<td>System Attributes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Applicability (autologous/allogeneic)</td>
<td>Both</td>
<td>Allogeneic</td>
<td>Allogeneic</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>Closed system</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Integrated concentration and washing</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Degree of automation</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Disposability, ready to use</td>
<td>Yes</td>
<td>Not yet</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Additional equipment/ utilities required</td>
<td>None</td>
<td>Tube welder and sealer</td>
<td>Air</td>
<td></td>
</tr>
<tr>
<td>System set-up and risk</td>
<td>Easy/low</td>
<td>Easy/low</td>
<td>Difficult/medium</td>
<td>Easy/medium</td>
</tr>
<tr>
<td>Tubing connections (flexibility)</td>
<td>Highly flexible, integrated</td>
<td>Flexible</td>
<td>Not flexible</td>
<td>Flexible</td>
</tr>
</tbody>
</table>

| Performance Attributes      |         |                    |               |              |
| Flow capacity (L/h)         | 1 – 750 | 25 – 1250          | 1 – 30        | <175         |
| Cell number capacity        | 1 – 100 x 10^7 | >100 x 10^7        | 6 – 30 x 10^7 | 20 – 100 x 10^7 |
| Scalability                 | High    | Appropriate for high volumes | Medium | Low |
| Operational labor           | Medium  | High               | Medium        | Medium       |
| Process/product monitoring capability | High   | High               | Low           | Low          |
| In-line metrics             | Pressure, flow | Pressure, flow, temperature | Speed, flow | Speed, flow, temperature |

Up to this moment and in the majority of clarification processes, cells are merely a by-product of the process, since the main goal is to clear them from the system [71, 73]. Therefore, several companies and research groups have been rethinking and redesigning TFF process in order to adapt this technique to suit the demands for cell-based therapies [69, 70], where cells are intended as the final product. Nonetheless, the impact of the TFF process parameters on the cells’ characteristics still needs to be assessed.
Continuous centrifugation (CC): CC can be operated in two different ways: counterflow or orthogonal. Systems such as Culturefuge® (Alfa Laval) or Unifuge® (Carr Centritech) use a continuous orthogonal flow, where fluid flows perpendicular to the centrifugal force. Unifuge® is a continuous, fully automated, single chamber and single use system able to process cell suspensions while maintaining high efficiency and keeping low shear stress. Culturefuge® is a disc-stack centrifuge for much larger bioprocessing scales which is not single-use, requiring cleaning and maintenance. This second system incorporates multiple intermittent solid discharges without a prior washing step, which is important for process streamlining to formulation and vialing. Cells are compacted in both systems, which diminishes washing efficiency (critical for cell therapy products). Separate washing steps could be performed to remove residuals, which is undesirable because it can further reduce cell recovery by 10–30% [68, 76, 77]. Furthermore, additional washes would increase the overall production time and cost for generation of therapeutic cells.

The other operation mode for CC is the counterflow. Two examples of continuous counterflow centrifugation systems already available are: Elutra® (Terumo BCT) and kSep® (KBI Biopharma) [78, 79]. Such systems allow cells to remain in suspension while supernatant and residuals are cleared. Elutra® is a fully automated single-use system with integrated washing capability and a wide range of chamber capacities (100 mL to 6 L). kSep® is a system that allows for cell recoveries above 80% while maintaining high cell viability (>90%). This is a system with big potential for DSP of cell therapy products, although it requires high investment: $200,000 to $700,000 for a system according to current good manufacturing practices (CGMP). Process development costs could also be high for processing the large number of cells required for meaningful development runs because scale-down modeling is not achievable.

1.3 Process integration

In the current environment of diverse product pipelines, rapidly fluctuating market demands and growing competition from biosimilars, biotechnology companies are increasingly driven to develop innovative solutions for highly flexible and cost-effective manufacturing. To address these challenging demands, integrated continuous processing, comprised of high-density perfusion cell culture and a directly coupled continuous capture step, can be used as a universal biomanufacturing platform.

Other industries such as the petrochemical, chemical, food or pharmaceutical have long converted from batch to continuous processing [80, 81]. The cell therapy industry will soon convert in the same way these ones did in the past. Despite the differences between these industries, the advantages of continuous manufacturing are always the same, including steady-state operation, small equipment size, high volumetric productivity, streamlined process flow, low cycle times and reduced capital cost [82]. One good example is the ongoing project at the Novartis – MIT Center for Continuous Manufacturing that targets a holistic redesign of the pharmaceutical manufacturing process to achieve fully integrated, continuous flow [83, 84]. The evolving competitive business environment is incrementally driving biotechnology industry towards a tipping point where existing barriers may be counterbalanced by the need for radically improved bioprocessing. Innovation in biotechnology has become more product- than process-centered [85]. Several authors have voiced
their concern about the long-term viability of the traditional manufacturing facility model based on multiple 10,000-20,000 L batch bioreactors and downstream trains [86]. The ability of such facilities to readily accommodate diverse biologics pipelines or fluctuating market demand is being more and more questioned. Furthermore, these facilities imply a huge initial investment, which will always be a main concern. The need for flexibility, consistent product quality, high process output and low cost is awakening the interest in continuous and integrated processes. Integrating the upstream and downstream unit operations will result in a significant simplification of the entire process train due to the elimination of non-value-added hold steps, dramatically shorter residence and cycle times, reduction of equipment size and overall facility minimization [85].

In terms of cell manufacturing, there is an obvious interest in integrated and continuous processes, for the same reasons that made other industries adopt these processes [85]. However, in terms of cell production for therapeutical uses, these concepts are not being applied yet, urging the need of studies integrating both up- and downstream processes.
2. **Aim of the thesis**

The main aim of this thesis was the development of a scalable integrated strategy for the up- and down-stream processing of hMSC. The first objective was focused in the implementation of a xeno-free bioprocess for cell expansion in a microcarrier-based stirred culture system. For this purpose, hMSC were cultured in environmentally controlled stirred tank bioreactors, and the impact of culture operation mode (continuous perfusion and semi-continuous) on cell growth, metabolism and cell characteristics was evaluated. A perfusion system with a cell retention device was also tested - the ATF™-1 (Alternating Tangential Flow) prototype, for its feasibility in supporting continuous perfusion of hMSC.

The second part of this work aimed at optimizing two steps of the downstream process workflow - cell concentration (i.e. volume reduction) and washing - using tangential flow filtration (TFF) technology. For the concentration step, the effect of the hollow fiber membrane pore size and operation mode (continuous and discontinuous) on cell recovery yields and viability was evaluated up to a volume reduction factor of 10. For the washing step (i.e. diafiltration), both continuous and a discontinuous operation modes were analyzed and compared in terms of impurities removal profile and cell recovery yields. All these experiments were performed with human foreskin fibroblasts (hFF), given their resemblance with human mesenchymal stem cells [109].

The best operating conditions were selected for the development of an integrated strategy for up- and down-stream processing of hMSC, comprising the steps of cell expansion, harvesting and clarification, cell concentration and washing. This strategy is outlined in Figure 2.1.

![Figure 2.1](image)

**Figure 2.1 – Aim of the thesis.** Integrated strategy for the up- and down-stream processing of hMSC.
3. Materials and methods

In this work, human BM mesenchymal stem cells (hMSC; STEMCELL™ Technologies, Grenoble, France), and human foreskin fibroblasts (hFF, ATCC collection, Cat nr CRL-2429) were used. All reagents used in the cell culture procedures were obtained from Gibco® Life Technologies™ (Carlsbad, USA), unless otherwise stated. hMSC were cultured both in planar (static, 2D) and in microcarrier-based stirred systems, as described in the following sections.

3.1 hMSC expansion in planar (2D) culture systems

hMSC were thawed in a 37 ºC bath and quickly added to 5 mL of culture Minimum Essential Medium Alpha (MEM-Alpha supplemented with 10% FBS and 2 mM L-glutamine) medium pre-warmed to 37 ºC. The cell suspension was then centrifuged at 300 g for 5 minutes, and the cell pellet was resuspended in MesenCult™-XF medium (STEMCELL™ Technologies) supplemented with 2 mM of L-glutamine. Cells were inoculated (3500 cell/cm²) into T-flasks (BD Biosciences, Bedford, MS, USA) pre-treated with MesenCult™-SF Attachment Substrate (STEMCELL™ Technologies) and placed inside an incubator at 37 ºC, in a humidified atmosphere containing 5% CO₂, according to manufacturer instructions. At day 5, the culture medium was partially exchanged (50%).

When 80% cell confluence was reached, cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) and detached with TrypLE™ Select during 5-7 minutes at 37 ºC. Following cell detachment, MEM-Alpha (supplemented with 10% FBS and 2 mM L-glutamine) was added to the cell suspension, followed by a centrifugation at 300 g for 5 minutes. The cell pellet was resuspended in MesenCult™-XF medium and inoculated into new pre-coated T-flasks, into spinner flasks or stirred tank bioreactors (as described in the following Section), or resuspended in a freezing solution (Iscove's Modified Dulbecco's Medium, IMDM; supplemented with 40% FBS and 10% DMSO) for cryopreservation.

3.2 hMSC expansion in stirred culture

3.2.1 Spinner flasks

Synthemax® II microcarriers (Corning®, New York, USA) were prepared according to the manufacturer instructions, resuspended in MesenCult™-XF culture medium (25% of the spinner flask working volume), and added to the spinner flask (Corning® spinner flasks, with a working volume of 125 mL) at a concentration of 16 g/L. In alternative, Cytodex™ 1 microcarriers (GE Healthcare) were used, at a concentration of 3 g/L. hMSC were inoculated in spinner flasks at a cell concentration of 0.25x10⁵ cell/mL. The vessel was then placed in an incubator at 37 ºC, in a humidified atmosphere containing 5% CO₂, under intermittent stirring (vessels were agitated gently for 30 seconds every 20 minutes).

After 5 hours, the remaining 50% of culture medium was added, and the agitation was set to continuous at 35 rpm. At day 5, the culture medium was partially renewed (50%). Through the culture
time, cell concentration, viability and percentage of colonized microcarriers was monitored. Culture supernatant was collected every day for analysis of metabolite concentrations and LDH activity (Sections 3.6 and 3.4, respectively).

Cell harvesting was carried out at day 7 – 8 (80% cell confluence). Agitation was stopped, and after microcarriers sedimented on the bottom of the spinner flask, the culture medium was removed, and cells were washed twice with DPBS. After microcarrier settling, DPBS was removed, TrypLE™ Select was added (50% of the working volume of the spinner flask on the last day of culture) and the spinner flask was placed in an incubator at 37°C, in a humidified atmosphere containing 5% CO₂, with continuous agitation at 35 rpm. After 10 minutes, MEM-Alpha medium was added, and the cell suspension was filtered using a 20 μm cell strainer (BD Biosciences) for MC removal. The filtered cell suspension was centrifuged at 300 g for 5 minutes, and the cell pellet was resuspended either in Dulbecco’s Modified Eagle Medium (DMEM) (supplemented with 10% FBS and 2 mM L-glutamine) to undergo to downstream processing (section 3.3) or resuspended in a freezing solution (IMDM supplemented with 40% FBS and 10% DMSO) for cryopreservation.

3.2.2 Environmentally controlled stirred-tank bioreactors

hMSC were inoculated (0.25x10⁵ cell/mL) in Biostat Qplus stirred tank bioreactors (Sartorius Stedim Biotech, Gottingen, Germany) with 0.4 L working volume. Data acquisition and process control were performed using MFCS/Win (Sartorius Stedim Biotech). The bioreactors were equipped with a pitched blade impeller (3 blades). The head plate has ports for pH and pO₂ electrodes (allowing online measurement and control of these parameters), ports for sampling tubes and for addition/removal of culture medium or MCs. pH was maintained at 7.2, and controlled automatically by CO₂ injection for culture acidification. The dissolved oxygen (DO) was kept at 4% oxygen tension (approximately 20% air saturation) and was controlled automatically by injecting a mixture of air and N₂. The aeration was performed in the headspace of the bioreactor, and was kept at 0.1 vvm.

Cell thawing and MC preparation were performed as described in section 3.2.1. For 5 hours, the bioreactors were operated with 50% of the final working volume, using an intermittent stirring profile: ON (1 minute at 60-65 rpm)/OFF (20 minutes). Afterwards, the remaining 50% of culture medium was added and stirring was turned on continuously at 40-60 rpm. At day 6 empty microcarriers were added to the bioreactor in a ratio of 2:1 (two parts of empty MCs for each part of colonized MCs), increasing the concentration from 16 g/L to 48 g/L. During the following 4 days, the stirring profile was kept intermittent: ON (5 minutes at 60 rpm)/OFF (1 hour). From day 10 onwards, stirring was set to continuous at 50rpm.

A summary of the experimental conditions used is presented in Table 3.1.
Table 3.1 – Summary of the experimental design, evidencing the used culture parameters

<table>
<thead>
<tr>
<th>Culture parameter</th>
<th>Biostat Qplus Stirred Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell concentration</td>
<td>0.25x10^5 cell/mL</td>
</tr>
<tr>
<td>Microcarriers</td>
<td>Corning Synthetmax II - 16 g/L (from Day 0 to Day 6) - 48 g/L (from Day 6 to Day 14)</td>
</tr>
<tr>
<td>Initial working volume</td>
<td>400 mL</td>
</tr>
<tr>
<td>DO</td>
<td>4% O_2 tension (20% air saturation)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 °C</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
<tr>
<td>Stirring profile</td>
<td>0 hours – 5 hours (priming): ON (1 minute at 60-65 rpm)/OFF (20 minutes) From 5 hours – Day 6: continuous stirring at 40-60 rpm Day 6 – Day 10 (migration): ON (5 minutes at 60 rpm)/OFF (1 hour) From Day 10 onwards: continuous stirring at 40-60 rpm</td>
</tr>
</tbody>
</table>

Three different operation modes were tested:

1) **Semi-continuous system**: a batch mode bioreactor, where punctual medium exchanges were performed: day 5 (50% v/v) and every 2.5 days from day 5 onwards (50% v/v) (or equivalently, 40% every 2 days).

2) **Continuous perfusion system**: a bioreactor coupled with a perfusion apparatus composed of a gravimetric control system and Biostat Qplus associated pumps (Sartorius Stedim). This system (previously described in the work of Tostões et al. [106]) was assembled similarly to the one presented on Figure 3.1, with the exception of the ATF™-1 device and its respective controller. This system operated without any cell retention device, taking advantage of the fact that microcarrier sedimentation speed is higher than the speed at which the exhausted culture medium was removed, allowing for a microcarrier-free outlet stream.

Weight in the inlet and outlet scales was monitored every 15 seconds by the gravimetric control system, which actuated on the pumps, turning them on or off, in order to maintain the volume of fresh culture medium fed to the bioreactor equal to the volume of exhausted culture medium removed, and according to the flow rate defined by the user. The user-defined parameter was only the desired perfusion flow rate, calculated accordingly to the dilution rate required, \( D \) (day\(^{-1}\)), calculated with equation 1:

\[
D = \frac{F}{V} \quad (1)
\]

where \( F \) is the perfusion flow rate (mL/day), and \( V \) is the working volume of the bioreactor at any given time (mL). On Table 3.2 are presented the dilution rates used in this work.
Table 3.2 – Dilution rates used in the bioreactor systems operating in a continuous perfusion mode

<table>
<thead>
<tr>
<th>Culture Time</th>
<th>Dilution rate (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 – 4</td>
<td>0</td>
</tr>
<tr>
<td>Day 4 – 5</td>
<td>0.5</td>
</tr>
<tr>
<td>Day 5 – 14</td>
<td>0.2</td>
</tr>
</tbody>
</table>

3) **Continuous perfusion system with a cell retention device**: a bioreactor adapted with a perfusion apparatus composed by three elements: ATF™-1 System (Repligen, Waltham, MA, USA), gravimetric control system and Biostat Qplus associated pumps (Sartorius Stedim). This system was assembled as shown on Figure 3.1:

![Figure 3.1 – Perfusion apparatus used in continuous perfusion cultures.](image)

This bioreactor was assembled and operated in the same manner as the one described in the previous section (Continuous perfusion system), with the addition of the ATF™-1 device (Figure 3.1). The Alternating Tangential Flow (ATF™) device comprises of a diaphragm which moves upwards and downwards in slow cycles, forcing the cell culture to move up and down through the ATF™ dip-tube. This diaphragm is operated by the Controller C1 (Figure 3.1), which has a pump that provides the pressure/exhaust cycles necessary to move the diaphragm. The operational pressures for this pump have been optimized in previous studies performed by the group in collaboration with Repligen for animal cell cultures in this system. The ATF™ device is also composed of a filtering membrane which operates on a dead-end regimen and, due to its pore size of 70 µm, does not allow for the passage of microcarriers. This allows both for microcarrier/cell retention, and for clarified supernatant removal. Since the pressure/exhaust cycles perform the backflush necessary to clean the membrane, this is
also a system with the inherent ability to reduce filter fouling. In Figure 3.2, a representative scheme of the ATF™ device is presented, both for the pressure and for the exhaust cycles.

![Figure 3.2 – Representative scheme of the ATF™-1 device operating in the exhaust and pressure cycles](image)

3.3 Downstream processing

3.3.1 Cell concentration (volume reduction)

The microcarrier-free cell suspension was concentrated using tangential flow filtration (TFF) in hollow fiber (HF) devices. This concentration was performed both in a discontinuous and in a continuous mode, as represented on Figure 3.3. Three different HF devices were tested, as summarized in Table 3.3. All the hollow fiber devices have a polysulfone membrane and were purchased from GE Healthcare Life sciences (Wilmington, USA).

In the discontinuous operation mode, the cell suspension is pumped from the feed vessel into the feed port of the HF device, through the interior of the fibers, out from the retentate port and back into the feed vessel. Molecules or particles (cells) larger than the pore size of the membrane are retained inside the fibers (retentate), whereas smaller molecules pass through the membrane pores (permeate). The permeate flux is controlled throughout the process by a valve/pump. When necessary, a valve was positioned at the retentate stream to restrict the tubing, increasing the transmembrane pressure (TMP). The pressure on the feed ($P_f$), retentate ($P_r$) and permeate ($P_p$) streams are monitored by SciPres Luer pressure sensors (SciLog, Wisconsin, USA). Weight and pressure data were acquired every 15 seconds.
Table 3.3 - Specifications of the hollow fiber devices used for the concentration step

<table>
<thead>
<tr>
<th>Device</th>
<th>Material</th>
<th>Surface area (cm²)</th>
<th>Pore size (µm)</th>
<th>Inner fiber diameter (mm)</th>
<th>Number of fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF A</td>
<td>Polysulfone</td>
<td>24</td>
<td>0.65</td>
<td>0.75</td>
<td>4</td>
</tr>
<tr>
<td>HF B</td>
<td></td>
<td>16</td>
<td>0.45</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>HF C</td>
<td></td>
<td>16</td>
<td>0.20</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Either Watson Marlow Model 120 S/DV 200 rpm pump (Watson-Marlow Pumps Group, Massachusetts, USA) or Tandem Model 1082 or 1081 peristaltic pumps from Sartoflow® Slice 200 benchtop crossflow system (Sartorius Stedim Biotech) were used. Pressure was monitored using SciPres luer pressure sensors on the inlet, outlet and permeate streams. Weight and pressure data were monitored and acquired every 15 seconds using the Sartoflow® Slice 200 benchtop crossflow system. A water flux test was performed before and after each experiment to determine the membrane’s permeability. The membranes were preconditioned with two membrane volumes of sterile DPBS (Gibco® Life Technologies™) before the concentration process.

3.3.1.1 Physical description of the TFF

In this section, some of the most important system variables are described. The transmembrane pressure (TMP) is defined as the difference between the pressure on the inner part of the hollow fibers (calculated by the average pressure between the inlet and outlet) and the pressure
on the outer part of the hollow fibers (the pressure in the permeate stream), and therefore is calculated as shown in equation 2:

\[
\text{TMP} = \frac{P_f + P_r}{2} - P_p \quad (2)
\]

where \( P_f \) and \( P_r \) are the pressure of the feed solution at the inlet and outlet of the device, respectively, and \( P_p \) is the pressure on the permeate side.

In this system, the flux is defined as the flow rate per unit of membrane area (\( J \)), in liters per hour per square meter (LMH, Lh\(^{-1}\)m\(^{-2}\)). The inlet flux, \( J \) (LMH), is therefore calculated as shown on equation 3:

\[
J = \frac{Q_{\text{inlet}}}{A} \quad (3)
\]

where \( Q_{\text{inlet}} \) is the feed flow rate (Lh\(^{-1}\)) and \( A \) the membrane surface area (m\(^2\)). Likewise, the permeate flux, \( J_p \) (LMH), can be calculated in the same way, according to equation 4:

\[
J_p = \frac{Q_p}{A} \quad (4)
\]

where \( Q_p \) is the permeate flow rate (Lh\(^{-1}\)) and \( A \) the membrane surface area (m\(^2\)).

For Newtonian and incompressible fluids, the flow inside a cylindrical tube can be described by the Hagen-Poiseuille law. The linear velocity of the cell suspension, \( v \) (ms\(^{-1}\)), inside the hollow fibers as following can be described by equation 5:

\[
v = \frac{Q}{A} \quad (5)
\]

where \( Q \) is the flow rate of the cell suspension inside one hollow fiber (m\(^3\)s\(^{-1}\)) and \( A \) is the cross section area of one hollow fiber (m\(^2\)). The shear rate, \( \gamma \) (s\(^{-1}\)), can then be calculated by equation 6:

\[
\gamma = \frac{8v}{d} = \frac{4Q}{\pi r^3} \quad (6)
\]

where \( d \) and \( r \) are, respectively, the inner diameter and inner radius of a single hollow fiber. The shear stress, \( \tau \) (Pa), is dependent on the viscosity, \( \mu \) (Pa.s), of the cell suspension, and can be calculated according to equation 7:

\[
\tau = \mu \gamma \quad (7)
\]

The flow regime was determined by calculating the Reynolds number (\( Re \)), according to equation 8:

\[
Re = \frac{\rho \cdot d \cdot v}{\mu} \quad (8)
\]

where \( \rho \) (Kg.m\(^{-3}\)) represents the fluid density.
3.3.2 Cell washing/diafiltration

The concentrated cell suspension was diafiltrated using tangential flow filtration (TFF), in the same HF device used in the previous step (concentration). The diafiltration was performed both in a discontinuous and in a continuous manner. On the continuous diafiltration, as schematized on Figure 3.4, a diafiltration solution is continuously added to the cell suspension (feed), while permeate is being removed. Both diafiltration solution addition and permeate removal were performed at the same flow rate, in order to maintain constant the volume of the concentrated cell suspension.

The discontinuous diafiltration operated with the same scheme, but in this case the diafiltration solution was added in bulk to the cell suspension (feed), both in equal parts. The system was then initialized, and the cells suspension filtered until it reached its original volume. In both systems, the filtration was stopped when the following condition was achieved:

\[ V_{DS} = 6 \times V_{CS} \]

where \( V_{DS} \) is the volume of diafiltration solution added and \( V_{CS} \) is the volume of the cell suspension, which is constant overtime.

The diafiltration was performed with DPBS, in order to observe the removal of contaminants from the cell suspension. A validation was also performed with human serum albumin (HSA).

Figure 3.4 – Continuous diafiltration in a hollow fiber device. A pump continuously adds a diafiltration solution to the cell suspension while permeate is being removed at the same flow rate.
3.4 Cell viability and lysis

Fluorescein diacetate-propidium iodide staining (FDA/PI): culture viability was assessed using the enzyme substrate fluorescein diacetate (FDA; Sigma-Aldrich, Steinheim, Germany) and the DNA-dye propidium iodide (PI; Sigma-Aldrich). Cells were incubated with 20 µg/mL FDA and 10 µg/mL PI in DPBS for 2-3 minutes and then visualized using fluorescence microscopy (DMI6000, Leica, Wetzlar, Germany). FDA is a non-polar, non-fluorescent compound which enters freely in cells. In viable cells, FDA is converted by intracellular esterases to a highly fluorescent compound (fluorescein). Therefore, active/healthy cells will appear green fluorescent. PI is a polar, red colored fluorescent compound which enters cells with low membrane integrity, intercalating with DNA. Therefore, dying/dead cells will be stained in red.

Trypan Blue exclusion method: total number of viable cells was determined after incubation with 0.1% (v/v) trypan blue dye in DPBS. Using a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany), cells with a damaged membrane (non-viable) will be stained in blue in presence of this compound.

Lactate dehydrogenase (LDH) activity: LDH is an intracellular enzyme, only present in culture supernatant when cell lysis occurs. LDH activity from the culture supernatant was determined by monitoring the rate of oxidation of NADH to NAD⁺ coupled with the reduction of pyruvate to lactate at 340 nm as described elsewhere [134].

3.5 Cell growth

Total cell concentration: to evaluate total cell concentration without the need of cell detachment from the growth surface, cells were lysed and the released nuclei were stained. Cells on microcarriers were resuspended in lysis buffer (1% Triton X-100 in 0.1 M citric acid), vortexed and incubated at 37°C for at least 2 hours. After incubation, the released nuclei were stained with 1% (w/v) crystal violet in lysis buffer and counted in a Fuchs-Rosenthal haemocytometer.

Expansion ratio or fold increase (FI): FI was evaluated based on the ratio \( X_{\text{max}} / X_0 \), where \( X_{\text{max}} \) is the peak of cell density (cell/mL) and \( X_0 \) is the lowest cell density (cell/mL).

Population doubling level (PDL): the PDL represents the total number of times that the cell population has doubled its concentration since inoculation, and it can be calculated using equation 9:

\[
PDL = \frac{\ln(FI)}{\ln(2)}
\] (9)

Specific growth rate (\( \mu_{\text{max}} \)): \( \mu_{\text{max}} \) was estimated using a first-order kinetic model for cell expansion during the exponential growth phase, according to equation 10:

\[
\frac{dx}{dt} = \mu_{\text{max}} X
\] (10)

where \( t \) (day) is the culture time and \( X \) (cell) is the value of viable cells for a specific time. The value of \( \mu_{\text{max}} \) was calculated by the slope of the growth curves during the exponential phase.
3.6 Metabolite analysis

Glucose (GLC), lactate (LAC) and glutamine (GLN) concentrations were analyzed in an automatic analyzer YSI 7100 Multiparameter Bioanalytical System (YSI Life Sciences®, Yellow Springs, Ohio, USA). Ammonia concentration was quantified enzymatically using a commercially available UV test (Roche, Basel, Switzerland). The specific metabolic rates ($q_{met}$) were determined as described in the literature [107].

3.7 Characterization of hMSC

3.7.1 hMSC differentiation potential

**Osteogenic differentiation:** 6x10$^4$ cells were seeded per well into 5 wells on a 24-well plate, and cultured in MEM-Alpha culture medium supplemented with 10% FBS. After cell confluence was reached (2 - 4 days), culture medium was removed from all wells, and 1 mL of StemMACS® OsteoDiff media (Miltenyi Biotec, Germany) was added to 3 of the wells, while 1 mL of MEM-Alpha (supplemented with 10% FBS) was added to the other 2 wells to serve as control of spontaneous differentiation. Cells were cultured for 3 weeks, changing (100% v/v) both culture media twice a week. After culture time, cells were washed with DPBS and fixed with 10% neutral buffered formalin during 30 minutes at room temperature. After fixation, cells were washed with distilled water and then stained with alizarin-staining solution (20 g/L).

**Adipogenic differentiation:** 6x10$^4$ cells were seeded per well into 5 wells on a 24-well plate, and cultured in MEM-Alpha culture medium supplemented with 10% FBS. After cells reached confluence (2-4 days), culture medium was removed from all wells, and 1mL of NH AdipoDiff Medium (Miltenyi Biotec) was added to 3 of the wells, while 1mL of MEM-Alpha (with 10% FBS) was added to the other 2 wells to serve as control. Cells were cultured for 2 weeks, while changing (100% v/v) both the control and differentiation culture media twice a week. After culture time, cells were washed with DPBS and fixed with 10% neutral buffered formalin per well for 30 minutes at room temperature. After cells were fixed, the wells were washed with distilled water and incubated with 60% v/v isopropanol during 3 - 5 minutes. After this period, isopropanol was removed, and cells were incubated with Red Oil solution (1.8 g/L) for 1 - 5min and then washed with DPBS. Finally, cells were incubated with Mayer's hematoxylin solution (Sigma-Aldrich) for 1 - 5min.

3.7.2 hMSC adhesion and proliferation capacity

hMSC were inoculated in 24-well-plates, previously coated with MesenCult™-SF Attachment Substrate. A cell inoculum of 5 x 10$^3$ cell/cm$^2$ was used and cells were cultured as described in Section 3.1 (hMSC expansion in planar (2D) culture systems). To evaluate hMSC adhesion capacity, the concentration of adherent and suspension cells was measured at 15, 30 and 60 minutes after inoculation.
To evaluate the proliferative capacity of hMSC, the cells’ growth profile was monitored for 13 days. In both experiments, cell concentration and viability was determined as previously described (Section 3.4, Trypan Blue exclusion method).

### 3.7.3 hMSC morphology and immunophenotype

**Immunocytochemistry:** hMSC (while adherent to the microcarriers) were washed with DPBS and fixed with a solution of 4% (w/v) paraformaldehyde (PFA, Sigma-Aldrich) and 4% (w/v) sucrose in DPBS at room temperature for 20 minutes. Alternatively, hMSC were detached and separated from the microcarriers following the harvesting protocol described in Section 3.2.1 (Spinner flasks), and then inoculated into a 24-well plate. Cells (attached to microcarriers or in 24-well plate surfaces) were washed with DPBS and incubated with a solution of 0.125% (v/v) fish skin gelatin (FSG, Sigma-Aldrich) and 0.1% (w/v) Triton X-100 in DPBS during 15 minutes at room temperature to permeabilize the cells. Cells were incubated for 2 hours at room temperature with the following primary antibodies: phalloidin (conjugated with Alexa Fluor® 488 dye) which binds to F-actin cytoskeleton molecules, anti-vimentin (a filamentous protein from the cytoskeleton), anti-N-cadherin (a transmembranar protein important for cell adhesion and responsible for adherent junctions between cells), anti-α-tubulin and 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining. Vimentin, N-cadherin, and α-tubulin samples were incubated with the red secondary antibody Alexa Fluor® 594. Cells were observed using fluorescence microscopy (DMI6000, Leica) and photographed using a digital camera (DFC 360 FX, Leica).

**Flow cytometry:** the hMSC suspension was washed with DPBS and a total of 2x10^5 cells were incubated for 20 minutes light protected at room temperature, with each of the following antibodies: CD44-PE, CD73-PE, CD90-PE, CD105-PE, CD166-PE, CD34-PE, CD45-PE, HLA-DR and isotope controls IgG1κ-PE, IgG2ak-PE and IgG2bk-PE (BD Biosciences). Cells were washed twice in DPBS, and analyzed in a CyFlow® space instrument. At least ten thousand events were registered per sample.

### 3.7.4 Apoptosis and metabolic activity assay

The percentage of apoptotic and metabolic active cells was evaluated using Apoptosis Assay Kit NucView™ 488 and MitoView™ 633 (Biotium, Inc., California, USA), following the manufacturer’s instructions. This kit contains the green fluorescent NucView 488 caspase-3 substrate (detects intracellular caspase-3) and the far-red fluorescent MitoView 633 mitochondrial dye (detects changes in mitochondrial membrane potential). After incubation with both reagents, cells were analyzed by flow cytometry (CyFlow® space, Partec GmbH, Münster, Germany). At least ten thousand events were registered per sample.

### 3.8 Protein and DNA quantification

Process samples were centrifuged at 300 g for 5 minutes at room temperature and the supernatant was kept at 4 °C for further analysis. Total protein concentration from the process
samples was determined using either bicinchoninic acid protein assay (Pierce Biotechnology, Rockford IL, USA), according to the manufacturer’s instructions or using direct detect (Merck Millipore) infrared (IR)-based assay. Protein samples were analyzed (using standard running conditions) by SDS-PAGE electrophoresis (XCell SureLock mini-cell system; Life Technologies), by loading the same protein amount onto NuPAGE™ Novex™ 4–12% (w/v) bis-tris precast polyacrylamide gels (Life Technologies). Proteins were visualized by Instant Blue (Expedeon, Harston, UK) staining.

Total double strand DNA content on the process samples was determined in 96-well plates using a PicoGreen™ dsDNA Assay kit (Life Technologies) according to the manufacturer’s instructions.
4. Results and Discussion

4.1 Upstream: production of hMSC

In the first part of this work, a xeno-free bioprocess for expansion of hMSC on microcarriers using stirred tank bioreactors was implemented. Two operation modes (continuous perfusion and semi-continuous) were tested and compared to spinner flask and planar (T-flask) systems.

4.1.1 Spinner flasks

In the first stage of the study of hMSC expansion, a stirred system (spinner flask) was tested to culture hMSC in microcarriers. The use of a serum-free, xeno-free culture media to expand human cells poses several advantages; it limits ethic (animal suffering) and scientific (batch-to-batch variations, contaminations with viruses, mycoplasms and prions) concerns [111], associated with the use of undefined animal components, such as fetal bovine serum. Therefore, hMSC were cultured both in MEM-Alpha medium with 10% FBS and also in the xeno-free culture media Mesencult™-XF (Table 4.1). Furthermore, two different microcarriers were also used in each spinner flask. Both these systems were compared to a planar (2D) culture control, performed as described in Section 3.1.

<table>
<thead>
<tr>
<th>Table 4.1 - Two different strategies for hMSC culture in spinner flasks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Culture media</strong></td>
</tr>
<tr>
<td>Mesencult™-XF</td>
</tr>
<tr>
<td><strong>Microcarriers (MC)</strong></td>
</tr>
<tr>
<td><strong>MC concentration</strong></td>
</tr>
<tr>
<td><strong>Specific available area</strong></td>
</tr>
<tr>
<td><strong>Culture time</strong></td>
</tr>
<tr>
<td><strong>Cell inoculum (X₀)</strong></td>
</tr>
</tbody>
</table>

hMSC were inoculated into both spinner flasks at the concentration previously optimized in the group: 0.25 x 10⁵ cell/mL. The cultures were processed as described in Section 3.2.1 (Spinner flasks), during 9 days, with a 50% (v/v) medium exchange at day 5.

The main results of this experiment are shown in Table 4.2. In both cases, cells successfully colonized more than 90% of the microcarriers, as it can be seen in Figure 4.1. However, it can be observed that cell growth was very limited, reaching maximum cell concentration values of 0.87x10⁵ cell/mL and 0.58x10⁵ cell/mL respectively for Spinner 1 and Spinner 2 cultures, which are lower when compared to the values reported in the literature for hMSC culture in spinner flasks with microcarriers (4x10⁵ cell/mL) [108].
Table 4.2 – Characterization of hMSC growth in stirred spinner flasks in two different conditions and in planar (2D) conditions

<table>
<thead>
<tr>
<th></th>
<th>Spinner 1</th>
<th>Spinner 2</th>
<th>2D culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_0$ Volumetric (x10^5 cell/mL)</td>
<td>0.25</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>$X_0$ Per cm² (x10^3 cell/cm²)</td>
<td>4.3</td>
<td>1.9</td>
<td>4.2</td>
</tr>
<tr>
<td>$X_{\text{max}}$ Volumetric (x10^5 cell/mL)</td>
<td>0.87</td>
<td>0.58</td>
<td>-</td>
</tr>
<tr>
<td>$X_{\text{max}}$ Per cm² (x10^3 cell/cm²)</td>
<td>15.1</td>
<td>4.4</td>
<td>13.1</td>
</tr>
<tr>
<td>Expansion ratio ($X_{\text{max}}/X_0$)</td>
<td>3.5</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Population Doubling Level (PDL)</td>
<td>1.8</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Productivity (x10^6 cell/L.day)</td>
<td>6.9</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Microcarrier colonization at day 9 (%)</td>
<td>93</td>
<td>91</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4.1 – Microcarrier colonization and cell viability of hMSC cultures in spinner flasks with two different strategies. Fluorescence images were obtained with FDA (live cells, green) and PI (dead cells, red). Scale bars: 100 µm.

Cells cultured in Spinner 1 (xeno-free culture medium) were evaluated in terms of their morphology by an immunocytochemistry assay. Adherent cells on microcarriers exhibited a high organization of the intracellular filamentous protein f-actin (Figure 4.2A). Furthermore, cells harvested from Spinner 1, maintained their potential to differentiate into the adipogenic and osteogenic lineages (Figure 4.3B), and no spontaneous differentiation was observed (data not shown). In terms of their identity, it was observed that hMSC maintained their immunophenotype, being negative for the hematopoietic markers CD34 and CD45 and showing high levels of the mesenchymal markers CD73, CD105 and CD166.
In terms of glucose and lactate concentration profiles, it can be observed the effect of changing 50% (v/v) of the culture media at day 5. This medium exchange translated in an increase in glucose concentration (Figure 4.3A) and a decrease in lactate concentration (Figure 4.3B). Nevertheless, on the last days of culture, glucose concentrations below 1 mM and lactate concentrations above 10 mM were observed, suggesting that additional medium exchanges should be performed after day 5.

Using spinner flasks (100 mL), dos Santos et al observed glucose starvation and ammonia accumulation at potentially inhibitory concentrations [108], while performing medium exchanges (25% v/v) every 2 days. This problem was solved firstly by performing additional medium changes and later by implementing a continuous perfusion strategy in stirred tank bioreactors (400 mL), with positive results [95].

Taking these results into account, it was logical to move into a more controlled system: stirred tank (ST) bioreactors. An environment where pH, temperature, agitation, dissolved oxygen and other parameters are fully controlled, could be of great advantage to hMSC expansion. In order to further increase volumetric productivity and cell expansion ratio, empty microcarriers are added to the culture.

---

**Figure 4.2 – Structural characterization and quality control of hMSC cultured under stirred conditions in a xeno-free, microcarrier system.** F-actin (green) organization in hMSC adherent to Synthemax® II microcarriers (A). Differentiation potential of hMSC into the adipogenic and osteogenic lineages after expansion in stirred conditions (B). Immunophenotype analysis of hMSC cultured in planar and stirred conditions (C). Scale bars: 50 µm.
(at day 6). This strategy would allow cells to migrate from the colonized microcarriers to the empty ones, further potentiating cell growth and expansion.

The ST bioreactor also allows for the implementation of other feeding strategies, such as perfusion. This could be of great advantage, since it was observed in the spinner flask cultures that glucose concentration reached very low values. Therefore, two feeding strategies were adopted in the ST bioreactors: semi-continuous (punctual medium exchanges) and continuous perfusion (continuous addition and removal of culture media), as schematized in Figure 3.1 in Materials and Methods.

![Figure 4.3](image)

**Figure 4.3** – Glucose and lactate concentration profiles in Spinner 1 throughout the hMSC culture. Glucose (A) and lactate (B) concentration profiles expressed in mM, through a culture time of 9 days.
4.1.2 Bioreactors

Physical-chemical variables such as pH, gas mix, agitation or temperature are more easily controlled in a bioreactor. hMSC expansion in this system was evaluated and the impact of two different operation modes - semi-continuous (punctual medium exchanges) and continuous perfusion (continuous addition and removal of culture media) - on cell’s growth and metabolism was evaluated.

4.1.2.1 Perfusion vs Semi-continuous

hMSC were expanded in bioreactors as described in section 3.2.2 (Bioreactors). A comparison of the main metabolic and growth parameters of the two tested operation modes (perfusion and semi-continuous) and 2D control cultures is presented in Table 4.3. Due to the numerous advantages already specified, both bioreactor systems resulted in a higher cell growth, when compared to a planar culture system and to a spinner flask system (Section 4.1.1 – Spinner flasks).

<table>
<thead>
<tr>
<th>Microcarrier-based culture in stirred tank bioreactors</th>
<th>Planar (2D) static culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcarrier concentration (g/L)</td>
<td></td>
</tr>
<tr>
<td>$X_0$ Volumetric ($x10^5$ cell/mL)</td>
<td>Days 0-6: 16 g/L</td>
</tr>
<tr>
<td>$X_0$ Per cm$^2$ ($x10^3$ cell/cm$^2$)</td>
<td>4.3</td>
</tr>
<tr>
<td>$X_{max}$ Volumetric ($x10^5$ cell/mL)</td>
<td>3.36</td>
</tr>
<tr>
<td>$X_{max}$ Per cm$^2$ ($x10^3$ cell/cm$^2$)</td>
<td>19.5</td>
</tr>
<tr>
<td>$\mu_{max}$ (h$^{-1}$)</td>
<td>0.016</td>
</tr>
<tr>
<td>Duplication time (day)</td>
<td>1.8</td>
</tr>
<tr>
<td>Expansion ratio ($X_{max}/X_0$)</td>
<td>13.44</td>
</tr>
<tr>
<td>Population Doubling Level (PDL)</td>
<td>3.7</td>
</tr>
<tr>
<td>Productivity ($x10^6$ cell/L.day)</td>
<td>22.4</td>
</tr>
</tbody>
</table>

On Figure 4.4 is presented the cell growth profiles (expressed in volumetric concentration) for both operation modes.

Figure 4.4 – Growth profile of hMSC cultures in environmentally controlled bioreactors, operated in two different modes. Volumetric concentration of hMSC during 14 days of culture in perfusion and in semi-continuous operation mode.
Both operation modes allowed to expand hMSC, exhibiting similar growth profiles. However, cell concentration on perfusion cultures was always higher than on the semi-continuous culture (Figure 4.4). This resulted in a higher overall expansion ratio (13.4 vs 11.1), a higher number of population doubling (3.7 vs 3.5) and a shorter duplication time, both from day 0 to day 6 (1.8 vs 2.6), as from day 7 to day 14 (5.8 vs 9.6). To be noted however that cell growth in the second phase of the culture (from day 6 to day 14) was lower in both systems. For example, in the perfusion bioreactor, the expansion ratio from day 0 to day 6 was of 8.8, and on the remaining days was only 1.5 (data not shown). During the migration phase (from day 6 to day 9), cells were transferring from colonized to empty microcarriers. Although good microcarrier colonization was obtained in this stage, cells could be migrating at the cost of growing slower. Culture time could also have had an influence; observing the control culture in T-flask (Table 4.3), a much lower expansion fold was also obtained in the second passage (data not shown). This could be indicative of hMSC senescence and loss of proliferation capacity, and is in accordance with the results obtained in both bioreactors. Maximum cell concentrations obtained in these cultures ($4 \times 10^5$ cell/mL) were lower than the ones previously reported by dos Santos et al. ($5 \times 10^5$ cell/mL) [95]. This difference may reflect the distinct cell origins (hMSC were isolated from the bone marrow of different donors), and the different culture conditions used in both studies, such as the microcarriers’ type and the culture medium formulation.

![Figure 4.5](image_url)

**Figure 4.5** – Microcarrier colonization and cell viability of hMSC bioreactor cultures in perfusion and semi-continuous operation modes. Fluorescence images were obtained using FDA (live cells, green) and PI (dead cells, red) (A) and were also used to assess cell viability and to calculate microcarrier colonization through culture time (B). Scale bars: 200 μm.
Cell viability (Figure 4.5A) and microcarrier colonization profile (Figure 4.5B) through the cultures were also analyzed. As it can be observed, at day 6 (before adding empty microcarriers) and at day 14 (final day of culture), microcarrier colonization was similar in both systems. However, significant differences after microcarrier addition could be observed; at day 8 of culture (day 2 of migration), 80% of the microcarriers were populated in the perfusion operation mode, while on the semi-continuous operation only 60% of the microcarriers were populated.

By constantly renewing the culture media using a perfusion operation mode, it was possible to guarantee a stable system regarding nutrients’ concentration and clearance of undesired metabolic products (Figure 4.6). Moreover, both lactate (Figure 4.6C) and ammonia (Figure 4.6D) concentrations were always below growth-inhibitory concentrations (<6 mM and <1 mM, respectively) [67].

During the first 4 days of culture, glucose consumption and lactate production rates were similar, since both cultures were performed under the same conditions until day 4. From day 4 onwards (beginning of perfusion operation) a decrease in both metabolic rates for both operation modes could be observed. However, this decrease was higher using the perfusion operation mode. Between the first (day 0 – day 4) and last intervals (day 10 – day 14), the decrease in the glucose consumption rate (Figure 4.7A) was of 3.3–fold in the perfusion operation mode and of 1.3–fold in the semi-continuous one. Similarly, for lactate production rate (Figure 4.7B), it was observed a 20.6-fold decrease in the perfusion operation mode, while this decrease was only 2.0-fold in the semi-continuous one.
continuous operation mode. A decrease in $q_{GLC}$ and $q_{LAC}$ after 3 days of culture of hMSC has also been reported before [67].

Glucose is the main ATP source in animal cells, either through oxidative phosphorylation (yielding 30-38 mol ATP per mol glucose) or through anaerobic glycolysis (yielding 2 mol ATP and 2 mol lactate per mol glucose). Therefore, the accumulation of lactate is associated with inefficient glucose metabolism [116-117]. For hMSC cultured in a semi-continuous operation mode in bioreactor, it was observed a lactate/glucose ratio ($Y_{LAC/GLC}$) of 2.0 ± 0.3 through the 14 days of culture (Figure 4.7C), suggesting the maintenance of an anaerobic glycolytic metabolism. However, when hMSC were cultured in a continuous perfusion mode, it was observed a drop on this yield, from 2.3 (day 0 – day 4) to 0.4 (day 6 – day 14). A metabolic shift occurred during the hMSC culture in a perfusion mode, from anaerobic glycolysis to oxidative phosphorylation, as demonstrated before in other studies with mammalian cells [118]. This meant that in a later stage of the culture, when hMSC were growing at a lower rate, glucose was being consumed more efficiently for energy production and cell maintenance, a phenomenon that was observed only in the perfusion culture.

This data is in accordance with the measured oxygen/glucose yields ($Y_{O2/GLC}$) observed (Figure 4.7D). As stated before, operating in a semi-continuous mode led to the maintenance of an anaerobic glycolytic metabolism, characterized by very low $Y_{O2/GLC}$. On the other hand, $Y_{O2/GLC}$ values while operating in a continuous perfusion mode were much higher, which is also in accordance with the lower $Y_{LAC/GLC}$ observed for this system.

Figure 4.7 – Specific metabolic rates of hMSC cultures in environmentally controlled bioreactors. Specific rates of (A) glucose consumption ($q_{GLC}$), (B) lactate production ($q_{LAC}$), (C) yield of lactate produced per glucose consumed ($Y_{LAC/GLC}$) and (D) ratio of oxygen consumed per amount of glucose consumed ($Y_{O2/GLC}$) both for the perfusion and the semi-continuous operation modes, throughout culture time.
After 14 days of culture in both bioreactor systems, hMSC were characterized in terms of identity (immunophenotype), potency (multipotent differentiation potential), as well as in proliferation and adhesion capacity. The expression of the main characteristic hMSC surface markers, as described in the literature [21], remained unaltered; more specifically, hMSC were negative for hematopoietic CD34 and CD45 markers, and displayed high levels of CD73, CD105 and CD166 mesenchymal stem cell surface markers (Figure 4.8A). hMSC cultured in a perfusion operation mode were plated on a 24-well plate to evaluate their proliferative capacity (Figure 4.8B). hMSC remained proliferative, achieving an expansion fold of 7.3 during 12 days of static culture.

Another characteristic of hMSC relies on their capacity in adhering to plastic surfaces [21]; therefore, hMSC cultured in the perfusion operation mode were evaluated in terms of their adhesion capacity: after 1 hour of culture in static conditions, 55% of the hMSC could adhere to the plastic surface on a 6-well plate (Figure 4.11C), and after 24 hours of culture all cells were adherent and already proliferating.

Figure 4.8 – hMSC characterization after expansion in bioreactors in perfusion and semi-continuous operation modes. Evaluation of the expression of the main characteristic hMSC surface receptors by flow cytometry (A), hMSC proliferation capability (B) and adhesion potential (C), cells’ morphology (D) and differentiation potential (E).
Moreover, hMSC morphology (immunocytochemistry assay, section 3.7.3) was also characterized. Cells harvested from the perfusion bioreactor were re-plated into 24-well plates and allowed to grow for two days. hMSC presented organized F-actin, and a homogeneous distribution of the mesenchymal filamentous protein vimentin and α-tubulin (Figure 4.8D). Finally, it was observed that hMSC harvested from both bioreactors were able to differentiate into the adipogenic and osteogenic lineages (Figure 4.8E).

4.1.2.1 Validation of the perfusion system using the Alternating Tangential Flow (ATF™) prototype

As reported on Chapter 3, a prototype of a perfusion system with cell retention was tested during this work, in order to assess cell growth limitations, cell detachment and death imposed by the prototype. This system was used to cultivate hMSC during 7 days and compared with a dip-tube based perfusion system and a semi-continuous system (Figure 4.9), described in section 4.1.2.1.

Through 7 days of culture, the bioreactor operating with the ATF™ prototype was able to reach a similar volumetric productivity (2.7x10^7 cell/L.day) as the other two operation systems (Figure 4.9A). Furthermore, the ATF™ device did not contribute with additional stress to the cell culture; until day 4 of culture, LDH accumulation profiles were similar in both perfusion systems, since perfusion only started at day 4. Nonetheless, from days 4 to 7 (while perfusion was operating) it was observed some increase in LDH accumulation for both cultures. When comparing both perfusion systems (ATF™ and dip-tube), it can be seen lower LDH release in cultures operated with the ATF™ device (Figure 4.9B). Therefore, the ATF™ prototype is a suitable solution for perfusion culture systems, providing similar cell growth as other traditional systems (e.g. dip-tube), without introducing any considerable stress to the culture.

Figure 4.9 – Comparison of hMSC culture using two types of perfusion operation (dip-tube and with ATF™ prototype) in an environmentally controlled bioreactor. Volumetric productivity, expressed in 10^7 cells per culture volume per day, of the two perfusion systems and on a semi-continuous system after cell expansion (A) and accumulation of LDH in culture media of both perfusion systems (B).
4.2 Downstream processing of hMSC

In this part of the work, different strategies for cell concentration and washing steps were evaluated. To accomplish both tasks, tangential flow filtration (TFF) using hollow fiber (HF) devices, with operating conditions already optimized in the group, were used. The main goal for the concentration step was to achieve a volume reduction factor (VRF) of 10, compare HFs with different pore sizes, and compare two different operation modes (continuous and discontinuous). Regarding the washing step, the objective was to compare two different operation modes (continuous vs discontinuous) while achieving protein and DNA clearance over 95%.

4.2.1 Concentration step

4.2.1.1 Impact of pore size in the performance of TFF

Process parameters used to perform TFF, have been previously optimized on the group [115], and are presented in Table 4.4.

| Table 4.4 – Previously optimized operation parameters for the TFF process. [115] |
|---------------------------------|-----------------------------|
| TFF parameter | Optimized parameter |
| Membrane material | Polysulfone |
| Initial cell concentration | $2 \times 10^5$ cell/mL |
| Wall shear rate ($\gamma$) | 3000 s$^{-1}$ |
| Permeate flux ($J_p$) | 250 LMH |

The impact of the pore size on the concentration performance was assessed using three different HF devices (Table 3.3, Chapter 3.3.1) and with human foreskin fibroblasts (hFF) as a cell model, given their resemblance with human mesenchymal stem cells [109]. Figure 4.10 summarizes the main results obtained from this experiment.

It is known that the membrane’s polysulfone material is prone to interact with the cells [114]. Therefore, cell recovery yield was estimated during the concentration process (Figure 4.10A). For a volume reduction factor of 10, higher pore sizes (0.65 µm) allowed for a cell recovery of 82%, while smaller pore sizes (0.45 µm) allowed for only 72% cell recovery. In both cases, cells maintained their viability above 95%, measured with the trypan blue exclusion method at each sampling point. Furthermore, when pore size was even more reduced (0.20 µm), the membrane became prone to fouling, leading to a complete clogging at a volume reduction factor of 4, hampering the process to continue. Effects of this phenomenon were reflected in the permeate flow, which started to drop instead of remaining constant through the process (Figure 4.10B) and in the transmembranar pressure (TMP), which raised up to a maximum of 0.4 bar (Figure 4.10C). For larger pore sizes (0.45 and 0.65 µm), both the permeate flow and the TMP remained constant during the whole process.
The membrane’s permeability was also determined before and after the process according to the manufacturers’ protocols, in order to assess fouling and clogging phenomena (Figure 4.10D). Membrane permeability decreased 10 - 15 % when using larger pore sizes (0.65 and 0.45 µm), being always higher than the recommended operating level of 9000 L.m⁻².h⁻¹.bar⁻¹. However, when a pore size of 0.20 µm was used, a clogging of the membrane was observed, being the membrane permeability reduced to 5% of its original value. A recovery of this HF was attempted by recirculating NaOH (1 M) during 30 minutes, increasing the permeability of this membrane up to 67% (7500 L.m⁻².h⁻¹.bar⁻¹) of its original value, which was still below the accepted limit of the manufacturer (Figure 4.10D).

Overall, HF A (24 cm² of surface area and pore size of 0.65 µm) was the most cost-effective device to perform the concentration process in this working scale (up to 0.25 L). It allowed to concentrate cells (10 fold volume reduction factor) with higher cell recoveries (over 80%, with viability higher than 95%), while keeping constant both the TMP and the permeate flux through the process. Since the inlet flux was kept constant (750 LMH), this HF also allowed for lower processing times, due to its higher surface area (24 cm² vs 16 cm²).

4.2.1.2 A continuous system for hMSC concentration in hollow fiber devices (feed and bleed)

At an industrial scale, picturing a future large-scale production of complex biotherapeutics, a continuous system where cells are being continuously fed and collected, could be advantageous. Therefore, an alternative approach on the concentration operation was tested, envisaging a system
working in a steady-state, with a continuous outlet of the target product: feed and bleed (see Figure 3.3B, Materials and Methods, Section 3.3.1). In continuous operations, the required time to reach steady state is usually negligible when compared to overall process time, leading to higher productivities than in discontinuous operations [112]. Moreover, the residence time of particles within the system is longer in discontinuous processing [113].

This continuous system was compared with the discontinuous one. Both processes were performed using shear rate and permeate flow conditions in agreement with the operating conditions optimized previously in the group. Cell density increased during the concentration step, up to a concentration fold of 6 in both processes. Cell viability was higher than 95% throughout the process, corroborating that cells have suffered minor shear stress in the concentration processes within the HF devices. Given that the process loop is operating continuously at a concentration factor equivalent to the final concentration of a batch system, the membrane area required is higher. We tested two different areas (24 and 50 cm²), which presented no differences in processing our working volume (data not shown).

Although the final cell recovery was similar on both operation modes, the concentration profile was distinct; in theory, continuous TFF is operated at the retentate concentration [110], yielding a concentrated product faster and for a longer period of time than on the batch operation (Figure 4.11A). Our experimental results also corroborated this trend, having reached higher concentration factors faster than in discontinuous operations. Although no flux decay occurred in continuous TFF, we have observed that similarly to the batch TFF, it is crucial to control the permeate flux control with a pump. A peristaltic pump controlling the feed instead of the permeate flow rate, leads to differences in cell recovery in 10 – 20% (data not shown).

Figure 4.11 – Continuous and discontinuous cell concentration. Schematic representation of two TFF operation modes – continuous and batch. Cell concentration profile throughout time (A). Cell characterization in terms of apoptosis, metabolic activity, DNA and LDH release profile before and after the concentration processes (B). Results are presented as a fold of change regarding cells before TFF. These proof-of-concept experiments were conducted with hFF.

No differences were observed between the two TFF operation modes regarding cell recovery yield; both allowed to recover more than 80% of cells with high viability (more than 95%) and metabolically active (Figure 4.11B). The observed 20% cell loss can be attributed either to cell death.
throughout processing, since an increase in the percentage of apoptotic cells as well as an increase in DNA and LDH release (fold change of 1.4 and 1.3) was observed (Figure 4.11B), or to cell retention inside the HF device.

4.2.2 Washing step

Effective removal of contaminants from a cell-based therapeutical product is required by regulatory agencies to ensure product safety [130], since trace amounts of host-cell DNA and proteins if injected into patients may potentially cause allergic reactions or even cause transfection of cells, resulting in tumor formation [131]. This was accomplished firstly by performing a discontinuous diafiltration (DF). This discontinuous DF was compared with a continuous DF system, as described in section 3.3.2 (Cell washing and diafiltration) and schematized in Figure 3.4. To study the contaminants’ removal profile, the DF (operated in continuous or discontinuous) was performed with DPBS as the washing solution; the main results of the DF operated in both modes are presented in Figure 4.12.

![Image of graphs showing protein concentration, cell recovery, DNA and LDH release, and SDS-PAGE analysis](image)

**Figure 4.12 – hMSC washing with DPBS, using TFF in HF devices.** Protein removal (A) and cell recovery (B) through 6 diafiltration volumes, LDH and DNA released during the process (C), SDS-PAGE analysis of protein samples from 1 up to 6 DVs for discontinuous and continuous DF: 5 µg (total protein) were loaded in each lane of the gel (D).

The results show that protein removal is effective in both operation modes (Figure 4.12A). Nevertheless, with discontinuous DF, a higher number of DVs (5-6) was needed to achieve the same
level of protein clearance than in the continuous process. To confirm this result, SDS-page was performed (Figure 4.12D). As expected, the total number of total proteins decreases along the increasing number of DVRs, i.e. protein is being efficiently removed from the concentrated cell suspension. As previously reported [132], continuous diafiltration showed to be more efficient and gentle than discontinuous DF [133]. In the discontinuous DF, lower cell recoveries (Figure 4.12B) were observed in the end of the process, as well as increased cell death (Figure 4.12C). On the other hand, continuous DF allowed for cell recovery yields of 70% of cells, with viability above 90%.

Overall, the continuous washing mode proved to be more suitable to remove impurities (higher protein clearance), providing a shorter processing time, allowing for a better cell recovery, while maintaining high cell viability.

In order to validate the washing process, hMSC were also diafiltrated using a formulation medium with 2.5% (v/v) of human serum albumin (HSA). Considering the previous results, concentrated hMSC suspension was continuously diafiltrated using 5 diafiltration volumes (Figure 4.13). It was observed a final cell recovery over 80%, with viability higher than 95%, without observing flux decay throughout the process.

![Figure 4.13 - Cell recovery yield during continuous DF (5 DVRs) using DPBS and a formulation media with 2.5% HSA.](image-url)
4.3 Integration of up- and downstream processes

Combining the results from the previous result sections, we have developed an integrated process of expansion, concentration and washing for hMSC (Figure 4.14).

After hMSC expansion using microcarriers in a stirred culture bioreactor using a perfusion system, cells were separated from the microcarriers using the operating conditions previously optimized in the group [115]. Cells were then concentrated and washed using the same hollow fiber device, with previously optimized conditions [115] of shear rate (3000 s⁻¹) and controlled permeate flux (250 LMH) using a permeate peristaltic pump; to integrate both processes, a discontinuous concentration and continuous washing were performed (Figure 4.14B).

hMSC were expanded during 14 days up to 13 fold (as described in Section 3.2.2 – Bioreactors) and concentrated up to a concentration factor of 10. After cell concentration, a continuous...
Diafiltration was performed using 5 diafiltration volumes of a formulation media, having recovered after the bioprocess 70% of highly viable hMSC (> 95%) (Figure 4.15C).

The integrated process showed not to impact cell morphology, since after plating hMSC (after cell expansion and after the bioprocess) could successfully re-acquire their typical spindle-like morphology, presenting organized intracellular f-actin, expressing α-tubulin and vimentin (Figure 4.15B). The expression of the main characteristic hMSC’s surface receptors, as described in the literature [21], remained unaltered after processing (Figure 4.15A). More specifically, hMSC were negative for hematopoietic CD34 and CD45 markers, as well as for HLA-DR and displayed high levels of CD44, CD73, CD105 and CD166 mesenchymal stem markers. hMSC maintained their ability in adhering to plastic surfaces and remained metabolically active, having reached a population doubling level (PDL) of approximately 3 after re-plating (Figure 4.15D).

**Figure 4.15 – Characterization of hMSC produced by an integrated process.** Expression of specific cell-surface antigens (CD166, CD105, CD73, CD44, CD45, CD34, HLA-DR) typical for hMSC, after cell expansion and at the end of the bioprocess (A). hMSC morphology after cell expansion and at the end of the bioprocess - immunofluorescence staining of f-actin (green), α-tubulin (red), vimentin (red), and cell nuclei (blue). Scale bars: 100 μm (B). Cell recovery yield after harvest, concentration and washing steps (C). Growth curves expressing the increasing cell number per volume of medium throughout the culture time of re-plated hMSC after cell expansion and at the end of the bioprocess (D).
The integration of the different processes allowed to decrease the equipment footprint, as well as the elimination of hold steps, especially by combining cell concentration and cell washing within the same HF device. All combined, the integrated process allowed for a faster process (1 hour after cell expansion, rather than typical 2 – 3 hours of separate processes), having been able to recover 70% of highly viable hMSC (> 95%), with no changes in terms of immunophenotype, proliferation capacity and multipotent differentiation potential.
5. Conclusion

In this work a scalable and integrated strategy for the expansion and downstream processing of hMSC was developed. A xeno-free microcarrier culture system was successfully implemented for the expansion of hMSC in stirred tank bioreactors. The continuous perfusion showed to be the most suitable operation mode for hMSC proliferation, allowing a higher cell expansion ratio (13.4) than the semi-continuous mode (11.2). In addition, hMSC display a more efficient (energetic) metabolism when cultured in continuous perfusion, as a metabolic shift from a glycolytic/lactic to an aerobic/oxidative metabolism was observed. The perfusion mode was also validated using the ATF™ cell retention device prototype, being obtained similar volumetric productivities (27x10^6 vs 25x10^6 cell/L.day).

To perform both concentration and diafiltration (washing) process, tangential flow filtration (TFF) technology was used. It was concluded that membrane pores larger than 0.45 µm were critical to perform the volume reduction step in hollow fiber (HF) devices, being beneficial for cell recovery yields and viability. hMSC were successfully concentrated up to a factor of 10, recovering 80% of viable cells, both in a discontinuous and in a continuous (feed-and-bleed) operation mode. The continuous concentration mode allowed however to operate longer with higher cell concentrations. Regarding cell washing, it was observed that when operating in a continuous diafiltration, a higher protein clearance (98%) was achieved while maintaining cell viability, when comparing with a discontinuous diafiltration.

Up- and downstream most suitable conditions were integrated, allowing for a shorter process time (1 hour of process time, rather than typical 2 – 3 hours of separate processes), having been able to recover 70% of highly viable hMSC (> 95%). At the end of the bioprocess, hMSC have conserved their characteristics, presenting high cell viability, maintaining their identity, potency and morphology.

Adult stem cells have a limited life span, and their characteristics change along the population doublings. Therefore, for cell therapy products, the advantages of a continuous, integrated process (up- and downstream) should be evaluated against current strategies where cell expansion is improved through medium renewal.

For the particular case of hMSC, by evidencing the feasibility and comparing the advantages of continuous and discontinuous operations, it was possible to design a more efficient process workflow. Higher productivities were achieved without compromising cells’ characteristics. This work represents a demonstration of the impact that continuous and/or integrated processing have on hMSC biomanufacturing, in terms of productivity and cell quality attributes.
6. References


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