Expansion of human mesenchymal stem/stromal cells (hMSCs) in bioreactors using microcarriers: lessons learnt and what the future holds.

Words: microcarriers, bioreactor, manufacturing, cell therapy

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Abstract

Human mesenchymal stem/stromal cells (hMSCs) present a key therapeutic cellular intervention for use in cell and gene therapy (CGT) applications due to their immunomodulatory properties and multi-differentiation capability. Some of the indications where hMSCs have demonstrated pre-clinical or clinical efficacy to improve outcomes are cartilage repair, acute myocardial infarction, graft versus host disease, Crohn's disease and arthritis. The current engineering challenge is to produce hMSCs at an affordable price and at a commercially-relevant scale whilst minimising process variability and manual, human operations. By employing bioreactors and microcarriers (due to the adherent nature of hMSCs), it is expected that production costs would decrease due to improved process monitoring and control leading to better consistency and process efficiency, and enabling economies of scale. This approach will result in off the shelf (allogeneic) hMSC-based products becoming more accessible and affordable. Importantly, cell quality, including potency, must be maintained during the bioreactor manufacturing process. This review aims to examine the various factors to be considered when developing a hMSC manufacturing process using microcarriers and bioreactors and their potential impact on the final product. As concluding remarks, gaps in the current literature and potential future areas of research are also discussed.

List of Abbreviations

AT, Adipose Tissue

BM, Bone Marrow

CFU-f, Colony Forming Units-fibroblast

CGT, Cell and Gene Therapy

CHMP, Committee for Medicinal Products for Human Use

CHO, Chinese Hamster Ovary

DMEM, Dulbecco's Modified Eagle Medium

FBS, Foetal Bovine Serum

hMSC, Human Mesenchymal Stem/Stromal Cell

hPL, Human Platelet Lysate

HSC, Hematopoietic Stem Cell

iPSC, induced Pluripotent Stem Cell

NSC, Neural Stem Cell

PBMC, Peripheral Blood Mononuclear Cells

SF, Serum Free Medium

STR, Stirred Tank Bioreactor

UCT, Umbilical Cord Tissue

XF, Xeno Free Medium

α-MEM, Minimum Essential Medium Eagle - alpha modification

1. Introduction

Cell and gene therapy (CGT) is a developing field of medicine that employs whole cells, or the products of cells (e.g. extracellular vesicles), as the therapeutic intervention, in contrast to using conventional small molecule pharmaceuticals or biopharmaceuticals (e.g. monoclonal antibodies). As the CGT field grows, multiple clinical trials using different cell types are currently underway. Aside from human mesenchymal stem/stromal cells (hMSCs), other cell types being used in these clinical trials include: T cells, hematopoietic stem cells (HSCs), neural stem cells (NSC), progenitors derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Couto et al., 2017; Fung et al., 2017; Trounson and McDonald, 2015).

Stem cells are one cellular candidate that has significant potential for the CGT field. Their prolonged self-renewal properties in conjunction with their differentiation capacity make them potential candidates for cell-based therapies. Stem cells can be classified based on their differentiation potential: (1) pluripotent (ESC or iPSC), (2) multipotent (such as HSC, NSC or MSC for instant) or unipotent (spermatogonial stem cells). Pluripotent stem cells can differentiate into all existent cell types. Multipotent stem cells are lineage specific. This cell type can only differentiate into cells from single specific lineage. This is the example of HSC from which all other blood cells are derived from. Lastly, unipotent stem cells can only differentiate into one cell type (NIH, 2016).

Research conducted with hMSCs has demonstrated their *in vitro* differentiation ability into various tissues such as fat, bone, cartilage and muscle, amongst others (J.-H. Lee et al., 2016; Munir et al., 2017; Tamama et al., 2008). Additionally, hMSCs have also exhibited immunomodulatory properties *in vitro*, i.e. they can modulate the fate of cells such as T-Cell, NK-Cell, B-Cell in the immune system (Hoogduijn, 2015; Nauta et al., 2006; Nauta and Fibbe, 2007).

These characteristics have been explored in hundreds of clinical trials globally, targeting a large spectrum of disorders from cardiovascular and neurodegenerative diseases to musculoskeletal

and immunological (Chang et al., 2014; Couto et al., 2017; Florea et al., 2017; Gupta et al., 2016; Jang et al., 2014; Liu et al., 2017; Milczarek et al., 2018; Panés et al., 2016; Pers et al., 2016; Tompkins et al., 2017; Wang et al., 2015). However, it is becoming increasingly clear that as we traverse the clinical trial stages, and the demand for cell number escalates with increasing numbers of patients, larger quantities of hMSCs are required for transplantation (from 1 to 2 up to 7 or 8 million of cells per kg of patient body weight) (Chang et al., 2014; Couto et al., 2017; Florea et al., 2017; Gupta et al., 2016; Jang et al., 2014; Liu et al., 2017; Milczarek et al., 2018; Panés et al., 2016; Pers et al., 2016; Tompkins et al., 2017; Wang et al., 2015; Want et al., 2012) and there is a concomitant need for large scale manufacturing systems that enable controlled and reproducible production of hMSCs.

Although hMSCs remain of interest as a cell therapy candidate, a new type of hMSC-derived product has emerged: cell-free therapy. As hMSCs have not been shown to engraft and differentiate *in vivo*, it has been suggested that their mechanism of action might be through paracrine action. Therefore a key challenge for hMSC production is to optimize bioprocesses to obtain two different products: (1) the hMSCs (for use as a cellular therapy) and the (2) secretome, which not only includes lipids, mRNA, growth factors and cytokines, but also exosomes and microvesicles (Vizoso et al., 2017). The secretome is of particular interest for the development of cell-free therapy products (Figure 1).

While both the hMSC and secretome can currently be manufactured for clinical trials, it is not known conclusively whether it is the hMSC or the secretome, or the combination of the two, that produces the intended therapeutic effect. Current and future research trends in this sector focuses on comparing the clinical effect of the secretome versus the cells, not only to study which product leads to the best clinical response, but also to determine the mechanism of action that is behind MSC-based and secretome-based therapies. Nevertheless, the production of both the hMSCs and cell-free products derived from hMSCs are of particular clinical and commercial interest, and their scalable manufacture remains a significant translational challenge. This review looks to provide a comprehensive overview of hMSC manufacture, with specific focus on the use of microcarriers and stirred-tank bioreactors (STRs), whilst highlighting gaps in the existing literature and emerging bioprocessing challenges that need to be addressed for scalable hMSC production to become reality.

(Insert Figure 1 Here)

2. Current production platforms for hMSCs

Over the last decade, the expansion of hMSCs has been performed using three different strategies: (1) monolayer cultures, (2) bioreactors (either hollow-fibre, packed bed, rotating wall vessels or stirred-tank or vertical wheel bioreactors with microcarriers) and (3) spheroids. Stirred-tank bioreactors have been successfully employed in the biopharmaceutical industry to culture CHO cells or VERO cells (Quesney et al., 2003, 2001; Trummer et al., 2006; Xing et al., 2009). Spheroid culture is the name attributed to the expansion of 3D cell aggregates. The rationale behind this approach is to mimic the three-dimensional environment that the cells experience *in vivo* (Bartosh et al., 2010; Wang et al., 2009). The challenge of this strategy is to expand hMSCs using large scale and

controlled bioreactors without causing detrimental effects on cells and their biological properties (Bonab et al., 2006a; Simões et al., 2013; Yang et al., 2018). Although the use of spheroids eliminates the dependency on microcarriers, this approach also includes several disadvantages. Together with the challenges faced during the spheroid manufacturing, spheroid size control and poor cellular fate control are some of the limitations of this approach (Fang and Eglen, 2017; Kapałczyńska et al., 2018; Wang et al., 2009).

In several hMSC-based clinical trials, the expansion process was carried out using monolayer cultures (Bartolucci et al., 2017; García-Arranz et al., 2016; Lamo-Espinosa et al., 2016). There are some disadvantages associated with expansion using monolayers, such as limited scale-up and high dependence on manual operators, which increases the risk of contamination and the overall cost of the process (Abraham et al., 2012; Chen et al., 2013; Shekaran et al., 2016; Simaria et al., 2014). Another disadvantage is the dependency on incubators to stabilize temperature and dO_2/pCO_2 concentrations. To avoid high concentration of metabolic products, the medium needs to be replaced manually, a time-consuming process which requires human handling which inevitably increases the process cost, or employ costly automated solutions which increase initial capital expenditure with relatively little return on investment. Moreover, many of these processes are open, which therefore require the use of expensive cleanroom facilities which significantly increase overheads and cost of production. All these reasons highlight the importance of developing large scale and controlled methods that automate the entire workflow, reducing the reliance on cleanroom capacity, need for skilled operators, and the overall production cost (Abraham et al., 2012; Chen et al., 2013; Shekaran et al., 2016; Simaria et al., 2014). As an extensive economic analysis of the manufacturing of hMSC cell-based and cell free CGT products is out of the scope of the current review, the reader is referred to several key reviews that explore this important aspect (Mizukami et al., 2018; Pereira Chilima et al., 2018; Russell et al., 2018).

While hMSCs cultured using spheroids have shown to possess improved angiogenesis and immunomodulatory properties (Follin et al., 2016; J. H. Lee et al., 2016) it is difficult to control the size of spheroids in culture (that can be up to 1 mm). Due to the mass transfer problems associated with these large 3D structures, it was reported that cell necrosis increased with increasing spheroid diameter (Groebe and Mueller-Klieser, 1996). However, necrosis has not been detected in spheroids with diameters between 200 and 360 µm (Lewis et al., 2016). In terms of bioprocessing, necrosis and other similar processes are highly undesirable not only because they will reduce total cell viability, but also because they have the potential to negatively impact neighbouring cells and cell quality. Aside from using microcarriers as a matrix to support cell growth and generate a single-cell suspension product (discussed later), a different approach adopted from the tissue engineering field includes the use of injectable microcarriers (Confalonieri et al., 2017; Zhang et al., 2016). The aim is to administer the microcarrier-cell in a way similar to using tissue-engineered scaffolds that can integrate and provide structural and biological support to repair and regenerate damaged tissue.

In addition to the expansion approaches considered above, there has been significant interest and research investigating the production of hMSCs in stirred-tank bioreactors. The bioprocessing industry has been using large scale stirred-tank bioreactors (STR) for decades for a wide variety of applications, with successful examples from the vaccines and monoclonal antibody production (Amanullah et al., 2004; Shukla and Thömmes, 2010; Tapia et al., 2016). Given the success of such platforms for Chinese hamster ovary (CHO) cells and *E.*coli production (Hewitt and Nienow, 2007; Nienow, 2006), such systems have been considered for use in CGT production, and specifically scalable hMSC production (Hewitt et al., 2011). There remain however significant challenges to adapt some of these technologies to meet the current demands in the CGT sector. The anchorage-

dependent nature of many cell types currently being considered as a cell therapy candidate, including hMSCs, presents technical challenges as the cells need a surface in order to grow. Their growth in STRs is therefore dependent on providing a suitable matrix (usually microcarriers) to which the cells adhere to and are then agitated. Another key difference is that for the first time, the cell forms the basis of the final product. This way of culturing cells raises additional challenges on the bioprocessing side because some unit operations may have detrimental effects on cell viability and functionality. These challenges include the optimization of the gassing strategy, establishment of the minimum agitation level required, optimization of the feeding regime amongst others. From a clinical and biological perspective, the focus is on cell identity and functionality; this remains the case from an engineering perspective. However, additional challenges such as process robustness and reproducibility need to be considered to enable scalability without changing the fundamental cellular properties.

The expansion of hMSCs using stirred-tank bioreactors and microcarriers is increasingly being considered as the primary, scalable method for hMSC production. Microcarriers are beads (with diameters around 100 to 250 μm) manufactured from different materials (such as polystyrene, dextran and cotton cellulose, among others) that provide a matrix on which anchorage-dependent cells can grow. The use of microcarriers for hMSC culture in stirred-tank bioreactors has been proven extensively and is described in more detail below (Carmelo et al., 2015; Chen et al., 2011; Dos Santos et al., 2014; dos Santos et al., 2013; Eibes et al., 2010; Goh et al., 2013; Jossen et al., 2016, 2014; Lam et al., 2017; Mizukami et al., 2013; Santhagunam et al., 2014; Tozetti et al., 2017).

In the specific case of hMSC production, key upstream process parameters and design considerations need to be determined to enable effective production. Some of these parameters and design considerations include: donor and cell source, dissolved oxygen (dO2), pH, temperature, medium exchange regime, type of microcarrier and agitation strategy amongst others. Although the vast majority of publications focuses on the upstream process, the downstream process must also be optimised, through the initial harvesting and subsequent purification steps. Harvesting is a long-standing challenge for hMSC based cell therapies. To separate cells from microcarriers three main strategies have been used: (1) enzymatic dissociation combined with high stirring speed (Nienow et al., 2014) inside of the bioreactor (Nienow et al., 2014) or using additional mixing devices (Santos et al., 2011), (2) enzymatic separation of the cells from the microcarriers using non continuous mixing (Caruso et al., 2014) and (3) enzymatic separation without any form agitation/mixing (Friedman et al., 2007; Shekaran et al., 2015). Each of these options are followed by a separation step that aims to retain microcarriers leading to a microcarrier-free cell suspension, ready for the subsequent downstream steps such as centrifugation or cryopreservation. There may be instances where additional downstream processing steps are required to ensure that any damaged microcarriers or microcarrier fragments are removed. Additional filtration steps may be required, or this separation could be achieved by other downstream processing units, e.g. centrifugation.

For cell-based therapies, the focus is to obtain a single-cell suspension, free from microcarriers and other processing reagents. This objective requires an effective harvesting strategy as described above. For therapies involving cell-free products (e.g. extracellular vesicles), the downstream processing challenges would be different. The product of interest can be collected from the culture supernatant, which avoids the need for cell/microcarrier separation; however, there will be additional challenges involved in concentrating and isolating the extracellular vesicle/exosome of interest from all others present in the culture medium. As reported several research groups (Lobb et al., 2015; Shtam et al., 2018; Tauro et al., 2012; Van Deun et al., 2014) different isolation methods seem to yield extracellular vesicle formulations with different degrees of purity. Additionally to

purity, the EV mRNA profile also seems to be affected by the isolation technique chosen (Van Deun et al., 2014). These downstream process challenges are more akin to traditional protein purification.

3. Overview of hMSC-microcarrier studies in stirred-tank bioreactors

Several clinical trials have published a dose response which necessitates the need for high-yield manufacturing processes (Galipeau and Sensébé, 2018; Golpanian et al., 2016; Murphy et al., 2013). Across the hMSC-microcarrier literature, a wide-range of yields have been reported as illustrated in Figure 2. This variability is probably due to a multiplexity of variables such as donor characteristics, initial cell properties, key differences in process parameters and culture conditions (described in Section 4). The majority of these studies were published at a working volume of 100 mL reporting cell yields from 1.5 x 10^5 cells/mL (Rafiq et al., 2013) or 1.52 x 10^5 cells/mL (Nienow et al., 2014) up to 4.2 x 10^5 cells/mL (Petry et al., 2016) 4.2 x 10^5 cells/mL (Tozetti et al., 2017) or even 8.8 x 10^5 cells/mL (Santhagunam et al., 2014) or 12.5 x 10^5 cells/mL (Jossen et al., 2016). Notably, the highest cell yields reported were obtained from hMSCs isolated from synovial membranes (Santhagunam et al., 2014) and adipose tissue (Jossen et al., 2016). When the scale increases (up to 3 L working volume), the highest cell yields reported were 4.4 x 10^5 cells/mL (Lam et al., 2017) and 5.3 x 10^5 cells/mL (Jossen et al., 2014). The studies conducted at the largest scale yielded 3.1 x 10^5 cells/mL (Schirmaier et al., 2014) and 1.9 x 10^5 cells/mL (Lawson et al., 2017) at a 35 L scale using a BIOSTAT STR® Plus 50L and a Mobius® 50L respectively (Figure 2). As cell yield is function of a plethora of parameters, it is extremely challenging to isolate a few who have the biggest impact on the final titter. To understand which factors, contribute with highest variance on the cell yield, robust mathematical tools such as principal component analysis should be performed.

It should be noted that for the majority of studies reported, an increase in scale seems to lead to a decrease in cell yield (Hewitt and Nienow, 2007). This drop will probably lessen as process understanding improves and there is a greater level of control. It should also be noted that most of the studies conducted have used adult sources of hMSCs, particularly those at the higher working volumes (>1L). It is important to highlight that working volume and the therapeutic target are closely related: if the objective is to have an allogeneic therapy, larger bioreactors are needed, which is not the case for the production of personalised autologous cell therapies. As hMSCs isolated from perinatal sources have demonstrated a higher proliferation ability that their adult counterparts (de Witte et al., 2017), it is likely that this will translate to larger-scale bioreactor studies.

All studies captured in Figure 2 focus on hMSC production with the cell acting as the therapeutic agent (Couto et al., 2020). However, with the increasing interest around exosome production, it is expected that manufacturing processes will be developed specifically optimized with this purpose in mind. To the best of our knowledge there are no publications that report how exosome production changes with biomass production. Hence, it is still premature to assume that process conditions that optimize cell growth also optimize exosome production. It was also reported that microcarrier-based expansion also seem to increase the production of cytokines when compared to monolayer cultures (Shekaran et al., 2015). This finding is particularly relevant from the perspective of developing a cell-free therapy.

(Insert Figure 2 here)

3.1. Impact of hMSC-microcarrier culture on potency

It was previously reported that the clonogenic ability of microcarrier-expanded hMSCs usually decreases by 13% when compared to monolayer-expanded ones. The same publication also showed that MSC expanded with microcarriers in bioreactors outperformed monolayer-expanded ones in terms of their CFU-F capacity, when the appropriate microcarrier was chosen (Lam et al., 2017). Another study also reported that the CFU-efficiency of BM-hMSCs was higher with expansion on microcarriers as compared to in monolayers (Heathman et al., 2016). In a different study, it was reported that microcarrier expanded hMSCs-derived from foetal origin showed higher expression of early osteogenic differentiation genes. The authors reported these findings as an advantage if the MSCs were used for bone regeneration applications (Shekaran et al., 2015). These results are aligned with the ones obtained by another research group, that reported improved adipogenic and osteogenic gene expression of BM-hMSCs expanded with microcarriers in comparison with monolayer-expanded ones (Sun et al., 2010). It was also suggested that the expansion method chosen (monolayer *versus* microcarrier-based) do not impact on the T-cell proliferation inhibition (Mizukami et al., 2016) under xenofree conditions. It should be mentioned that this study used cells in a lower passage (P4) and it is not possible to know whether the results would be similar if cells from a higher passage were used. Similar results were reported in another study that at a ratio of 1:5 (hMSCs:PBMC), microcarrier-expanded BM-hMSCs showed improved T-cell proliferation inhibition using 15% FBS supplemented α-MEM (Caruso et al., 2014). These results seem to suggest that different manufacturing options lead to hMSCs with different properties and potency assays are critical to ensure quality of the batches produced.

4. Key parameters to control/modulate during microcarrier-based expansion in bioreactors

The significant disparity in cell yield between hMSC-microcarrier STR studies demonstrated in Figure 2 are probably due to the differences in key culture parameters as well as the properties of cells and of the raw materials. Harvesting efficiency is also expected to impact on final cell yield and quality. The expansion of hMSCs with microcarriers using STRs is a multi-stage process that includes many different variables which need to be monitored and controlled throughout. This control is critical, not only in order to obtain high cell densities but also for the maintenance of potency of the desired cell populations (Table 2). The key culture parameters which need to be monitored, controlled and optimised are described in detail below.

4.1. hMSCs source and isolation method

As alluded to previously, hMSCs can be isolated from multiple different sources, either adult or perinatal. MSCs isolated from different sources present different bioprocessing considerations and have exhibited different functional characteristics (Table 1). A higher proliferation capacity of perinatal sources of hMSCs for instance, has been reported compared to adult ones (Jin et al., 2013; Simões et al., 2013). In addition to growth kinetics, hMSCs from different sources may also have different potency. It was reported that human umbilical cord-derived MSCs (UCT-hMSCs) promoted enhanced T-cell proliferation inhibition compared to human bone marrow-derived MSCs (BM-

hMSCs) (Bárcia et al., 2015). These findings were supported by another research group that also reported that adipose tissue-derived hMSCs (AT-hMSCs) exhibit a similar immunomodulatory profile when compared with placenta derived-MSC (PD-hMSCs) and UCT-hMSCs. Other authors have published differences in vasculogenic properties of different hMSC sources (Du et al., 2016) as well as differences at the differentiation and gene level (Du et al., 2016; Kwon et al., 2016). These findings reinforce the need for screening which cell source(s) would improve clinical outcomes prior to embarking on establishing a scalable bioprocess. This screening is important because in addition to the differences in cell performance or functionality, the hMSC source has an impact on the bioprocess.

Isolating hMSCs from tissues require either a form of processing to effectively generate a population of cells *in vitro*; or isolation can be performed using enzymes, mechanically or using an explant method. This step will increase the cost of the process and the time required to perform all of the isolation steps (Fazzina et al., 2015; Mori et al., 2015; Timmins et al., 2012). The success of the whole manufacturing process depends also on the isolation steps due to their impact on cell quantity and quality.

Table 1-Summary of adult and perinatal MSC characteristics**.**

	Adult (AT and BM)	Ref.
√	Can be isolated from materials that used to be discarded (such as liposuctions)	(Schneider et al., 2017)
X	MSC concentration decrease with age	(Choudhery et al., 2014)
	Perinatal	**Ref.**
√	Higher proliferation ability compared with adult ones	(de Witte et al., 2017)
√	Can also be isolated from tissues that would be discarded otherwise	(Secco et al., 2008)
√	Painless collection procedure	(Nagamura-Inoue and He, 2014)
√	Potentially improved immunomodulatory properties	(Bárcia et al., 2015; Li, 2014)
√	High rates of isolation (except for CB)	(Secco et al., 2008; Simões et al., 2013)
√	Lower probability of accumulating mutations resulting from aging or exposure to virus or toxins	(Capelli et al., 2011)

4.2.**Temperature and pH**

Temperature is one of the key parameters to be controlled to ensure optimal cell growth. This parameter is routinely set to 37 °C. While in spinner flasks, this temperature is achieved passively through the use of incubators, with STRs, it is maintained by a temperature sensor and control system which ensures the temperature remains at a certain setpoint (Chen et al., 2015; Dos Santos et al., 2014; Hupfeld et al., 2014; Jossen et al., 2016, 2014; Lam et al., 2017; Lawson et al., 2017; Mizukami et al., 2016; Rafiq et al., 2013; Schirmaier et al., 2014). Several groups have investigated the use of STRs for microcarrier-based hMSC expansion examples, with different bioreactors using

different mechanisms to maintain the culture temperature. The Biostat B Plus® (5L) from Sartorius (Rafiq et al., 2013) and Celligen® from New Brunswick (Mizukami et al., 2016) uses a water jacket, the DASbox® uses aIndividual temperature control with liquid-free heating and cooling (Peltier), whilst in the UniVessel ® (Sartorius) (Schirmaier et al., 2014) and Mobius® 3L, 50L (Applikon) (Lawson et al., 2017) heating blankets are used to keep the temperature at 37 °C (Heathman et al., 2019).

Although 37 °C is the temperature chosen by default, decreasing the temperature during the exponential phase has been shown to increase protein production (Furukawa and Ohsuye, 1998) or fragments of antibodies using some CHO cell lines (Schatz et al., 2003). Future research activity may focus on investigating the impact of temperature in exosome production as well as in the whole secretome. Such an investigation is especially critical if the goal is to establish a cell-free product.

Human MSCs use glucose as a carbon source while lactate and ammonia are formed as metabolic products, during the expansion process (Pattappa et al., 2011). The accumulation of the metabolic products causes cell growth inhibition as demonstrated previously (Schop et al., 2009). While pH is not easy to control in static cultures, STRs use sensors and other methods to prevent pH from going to levels that inhibit cell growth or even cause loss of functionality. Usually CO_2 (Chen et al., 2015; Goh et al., 2013; Rafiq et al., 2017, 2013; Santos et al., 2011; Tozetti et al., 2017), a mixture of air and CO_2, N_2, O_2 (Lam et al., 2017) are used for pH control and sodium bicarbonate (Dos Santos et al., 2014; Lawson et al., 2017; Mizukami et al., 2016) is used as a buffer. It is possible to find different working ranges for pH across the literature: 6.7 to 7.2 (Rafiq et al., 2013) or between 7.2 to 7.4 or even between 7.2 and 7.8 (Jossen et al., 2014; Lawson et al., 2017). While trying to optimize the expansion of BM-hMSCs in suspension under serum free conditions using CultiSpher-S microcarriers, it was reported that during the first 8 hours of the attachment period, pH 8.0 optimized the percent attachment when compared to pH 7.1 or 7.6 (Yuan et al., 2014). As mentioned previously, when studying the impact of high concentrations of ammonia and lactate on hMSC growth and the conclusion was that growth inhibition started once a medium concentration of 2.4 mM of ammonia or 35.4 mM of lactate was reached. However, these conditions did not adversely affect the hMSC differentiation capacity (Schop et al., 2009). Although the study investigated the impact of key metabolites on BM-hMSC growth and differentiation capacity, further studies are required to investigate the impact of metabolite concentrations on other aspects of cell functionality and to test the impact on other hMSC sources. Due to the lack of pH control in the aforementioned study, it is not possible to conclude whether the effect is driven by the high concentrations of ammonia/lactate, a change in pH or a combination of both. Another culture parameter that may play an impact is osmolarity as it is expected to increase with higher concentrations of metabolites. Additionally, there is still a need to investigate whether the metabolite profile changes whether the cells are grown in a monolayer system or under agitated bioreactor conditions, particularly as there would be an expectation that under agitated conditions, the culture environment would be significantly more homogeneous.

4.3. **Oxygen Supply and Aeration Strategy**

While cells are routinely expanded in 'normoxic' conditions (atmospheric O_2 conditions of 20% which in solution at equilibrium is designated 100% dO_2), *in vivo* it has been demonstrated that the cells experience low oxygen concentrations in certain niches and tissues (Bizzarri et al., 2006; Chow et al., 2001; Harrison et al., 2002). Knowing that, several authors have expanded hMSC under "hypoxic" conditions (2-5% O_2 v/v in the incubator atmosphere, or 10–25% dO_2). A significant advantage of a stirred-tank bioreactor system over traditional monolayer culture systems is the

extensive process monitoring and control capability. Such systems allow a greater level of control over a range of parameters, and it is becoming increasingly clear that oxygen concentration plays a significant impact on cell growth and functionality.

Trying to mimic what happens *in vivo,* it was reported that BM-hMSCs expanded under hypoxia showed improved growth rate and clonogenic ability, when compared to normoxia (Dos Santos et al., 2010) due to reduced oxygen consumption and reduced ROS production (Bonab et al., 2006a). Similar results were obtained by another research group, that reported that AT-hMSC expanded in hypoxic conditions kept their differentiation potential untouched while expressing lower levels of apoptosis, when compared to normoxia-expanded AT-hMSCs (Feng et al., 2014). It was also published that under hypoxic conditions, BM-hMSCs proliferate faster and have a higher colony forming potential when compared to cells grown under normoxic conditions (Krinner et al., 2009). The authors also reported that to "prime" the BM-hMSCs towards a chondrogenic lineage, an oxygen concentration in the gas phase of 10-11% O_2 (50-55% dO_2) was required (Krinner et al., 2009). Similar findings were recently reported in another study, revealing that oxygen impacts morphology, growth kinetics, differentiation ability and metabolic profile (Wang et al., 2018). The study found that, under hypoxic conditions, cells became smaller and had a lower population doubling time. It was also reported that asparagine and glutamine levels in the culture supernatant of hypoxically cultured hMSCs, were significantly lower than under normoxic conditions, suggesting that cells were following different metabolic pathways. It should be also mentioned at this point that the experiments conducted in monolayer using incubators do not usually offer great control of dO_2 (Heathman et al., 2019). The use of different medium formulations used, donor to donor variability from a cell perspective and the fact that any reduction in the concentration of oxygen in the gas phase is called "hypoxic" though each different concentration leads to a different dO_2 in the media might help to explain the inconsistent impact of the latter on hMSC growth kinetics.

While most of the studies reporting the impact of oxygen on proliferation ability are using adult sources of hMSC, this trend seems to be followed also by perinatal ones (UCT-MSC in this case) as published recently. Although the oxygen level impacts growth kinetics, it does not appear to impact the immunophenotype of UCT-hMSCs (Widowati et al., 2014). With respect to bioprocessing, not only is it important to establish the optimal oxygen concentration, but the method of controlling gaseous exchange in the system is also critical. It is possible to deliver gases directly into the culture medium (sparging) or into the bioreactors' headspace. Most studies have used the latter approach but recently, it was shown that sparging gases directly in the medium had a negative impact in BM-hMSC growth, mostly probably due to poor cell attachment to microcarrier rather than cell damage that occurs in free suspension culture such as CHO when bubbles burst at the liquid surface (Nienow, 2006). One way of minimizing the impact of cell-bubble attachment in the latter case is to use surfactants such as Pluronic™ F68 (Nienow, 2006) that has the role of reducing the hydrophobicity of the cell surface. However, it was suggested that the same mechanism make it more difficult for cells to attachment to microcarriers (Farid et al., 2000), which led to a negative impact on cell culture performance (Heathman et al., 2018). Although sparging has a negative effect on cell growth, neither cell viability or immunophenotype were affected (Heathman et al., 2018).

4.4. **Bioreactor Seed Train**

To have enough cells to seed the bioreactor, hMSCs are routinely expanded in monolayer culture. It has been previously reported that hMSC proliferation and differentiation capacity decreases when cultured in monolayer for prolonged periods (Alt et al., 2012; Bonab et al., 2006b; de Witte et al., 2017). It was reported that the decrease in proliferation is linked to the decrease in

telomere length which is responsible for *in vitro* aging (Bonab et al., 2006a). It was also recently reported that after several passages, hMSCs begin to lose their ability to suppress T-cell proliferation (de Witte et al., 2017). The ideal scenario is to maintain the cells at the lowest number of doublings possible to avoid functionality loss. To seed the bioreactors with hMSCs, different authors have adopted different strategies: while some tend to keep the passage number low, P2/P3 (Jossen et al., 2016; Nienow et al., 2014; Petry et al., 2016; Schirmaier et al., 2014), others have chosen to seed the bioreactor with high passaged hMSCs, up to P9/P10 (Lam et al., 2017; Lin et al., 2016). It must be noted that although research groups have been reporting the passage number, population doublings should also be reported because it adds another layer of information about the *in vitro* aging. While the effect of expansion in monolayer has already been published by several researchers (Bonab et al., 2006b; de Witte et al., 2017; Heathman et al., 2015), the effect of prolonged expansion using microcarriers in a stirred-tank bioreactor is still not fully understood. In fact, in monolayer, not only the growth kinetics but also the immunomodulatory properties have been shown to decrease with the increasing number of passages that the cells undergo during the expansion process (Bonab et al., 2006b; de Witte et al., 2017; Yang et al., 2018). It was shown that the immunomodulatory properties do not seem to change during expansion using xeno-free media in spinner flasks (Tozetti et al., 2017). This finding suggests that the immunomodulatory properties are not changed by expanding hMSCs in STRs. However, the impact on the immunomodulatory properties due to the expansion platform chosen, remains inconclusive. The same study also reported a decrease in CD105 expression post STR-based suspension, which had been reported in previous studies too. Some authors suggest that the shear stress or bulk-liquid turbulence due to mechanical agitation might be affecting cell receptors, in this case, CD105 (Dos Santos et al., 2014; Mizukami et al., 2016; Tozetti et al., 2017). However, there are not any studies which directly substantiate such speculation.

4.5. **Medium Formulations**

Several options can be considered when choosing the medium formulation to be used during the manufacturing process. Fetal bovine serum (FBS) supplemented medium has been used by many groups (DMEM (Chen et al., 2015; Heathman et al., 2016; Thomas R J Heathman et al., 2015; Lawson et al., 2017; Rafiq et al., 2013; Yuan et al., 2014), α-MEM (Caruso et al., 2014; Chen et al., 2015; Lam et al., 2017; Lawson et al., 2017; Lin et al., 2016; Schop et al., 2010; Shekaran et al., 2015; Takahashi et al., 2017; Tozetti et al., 2017)). Typically the FBS ranges from 5% (Jossen et al., 2016; Kaiser et al., 2013; Schirmaier et al., 2014) up to 15% (Caruso et al., 2014; Schop et al., 2010; Tozetti et al., 2017) of the total medium volume. Although FBS contains growth factors that promote cell growth, it also offers several disadvantages. Ethical issues associated with the collection procedure, batch to batch variability, limited global GMP supply and potential contamination with prions are amongst the biggest concerns with FBS use (Brindley et al., 2012; Wappler et al., 2013). For these reasons, regulatory agencies such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have developed specific guidelines for FBS usage in CGTs (EMA Committee for Medicinal Products for Human Use (CHMP), 2013). Despite of these disadvantages, serum is still used in the manufacturing process of several hMSC-based clinical trials (Cimino et al., 2017).

To avoid using serum, two options have emerged: (1) to use other complex solutions to replace the action of serum such as human platelet lysate (hPL) (Antoninus et al., 2015; Bieback, 2013; Bieback et al., 2009; Doucet et al., 2005) and human serum (autologous or pooled) (Aldahmash et al., 2011; Bieback et al., 2009; Kocaoemer et al., 2007), or (2) serum/xenofree medium (SF/XF) (Carmelo et al., 2015; Eibes et al., 2010; Rafiq et al., 2017; Santhagunam et al., 2014; Santos et al., 2011; Sousa et al., 2015; Tan et al., 2015). Both hPL and human serum are blood

derived products that are manufactured in different ways. Human serum is obtained allowing the peripheral blood to clot, followed by one or more centrifugation steps. For hPL, the manufacturing process starts with the isolation of plasma from blood that is usually submitted to freeze and thaw cycles after leukocyte removal (Bieback et al., 2009).

It was reported that hPL and SF/XF based medium resulted in higher proliferation levels of AT-MSC and BM-MSC (Oikonomopoulos et al., 2015). The same study reported that hMSCs expanded with DMEM with 10% of hPL exhibited lower immunosuppressive capability when compared to SF/XF supplemented DMEM. Other groups have found that hMSCs expanded using SF medium also exhibit enhanced clonogenic potential (Heathman et al., 2016). The study also found that the secretory profile of SF/XF-expanded MSCs was consistent which was not the case with the serum-supplemented medium (Swamynathan et al., 2014). This is an important advantage of using a chemically defined SF/XF medium to obtain a more consistent product. Having to change between lots of serum during a manufacturing process would require an extensive comparability study and consume significant time and resource.

4.6. **Bioreactor**

While the aim of this review is to focus on hMSC expansion in large scale STR platforms using microcarriers, it is important to recognise that are other options besides STRs including rocking motion (Jossen et al., 2016) bioreactors, hollow fiber bioreactor (Jones et al., 2013) and packed bed bioreactors (Osiecki et al., 2015). The reader is referred to a recent review for a comparison of different bioreactor platforms (Rodrigues et al., 2011). Another parameter that must be considered if pursuing a STR-microcarrier based expansion process for hMSCs is the type of STR system to use. Several different commercially available STR systems are currently available and many have been used for hMSC-microcarrier culture including the Biostat B (Sartorius) (Chen et al., 2015; Goh et al., 2013; Lam et al., 2017; Rafiq et al., 2013; Schirmaier et al., 2014), Biostat Q Ambr®15 (Sartorius) (Rafiq et al., 2017), Ambr®250 (Sartorius), BioFlo®/CelliGen® (Eppendorf) (Dos Santos et al., 2014; Mizukami et al., 2016), DASGIP (Eppendorf) (Dos Santos et al., 2014), UniVessel (Sartorius) (Jossen et al., 2014; Schirmaier et al., 2014) and Mobius® (Merck) (Lawson et al., 2017), amongst others. Each of these STRs differ with respect to the vessel geometry, working volume, impeller design, and some offer different levels of control.

Little research has been undertaken comparing the growth of hMSCs on microcarriers in different STR systems. However, given the potential for different fluid flow patterns and energy dissipation rates at similar agitator speeds in different STRs, there may be observable differences with respect to cell growth and potentially cell functionality. On the other hand since so many different types have been successful, perhaps the choice is not so important. Interestingly, Nienow et al (2016) found very similar culture performance with the same cell donors and microcarriers in four different types of bioreactor at 100% dO_2 provided each was operated at an agitator speed which just kept all of the microcarriers in suspension. These bioreactors were 15 mL ambr, 125 mL spinner flask, 250 mL Dasgip bioreactor and the 5 L (2.5 L working volume) Sartorius Stedim bioreactor. Though each required notably different power inputs to ensure suspension, cell quality was not compromised. It should be mentioned however that increasing the scale seems to lead to lower cellular yields, possibly because the scale up strategy chosen (for example, constant tip speed, which significantly lowers the specific power) leads to inadequate suspension. This result also suggests that the choice of bioreactor may not be important provided it has a flexible agitation capacity able to provide a sufficient specific power to ensure adequate microcarrier suspension and good control of such parameters as temperature, dO_2 and pH. This observation suggests a suitable

scale-up strategy once optimisation at small scale of such parameters as the choice of microcarrier, media and the like have been undertaken.

Table 2- Summary table with bioreactor characteristics and operator conditions used in for hMSC expansion.

Bioreactor (Manufacturer)	Working Volume (L)	Impeller type [II]	Vessel H/d (mm) [II]	Vessel Characteristics	Ref.
Ambr® 15 (Sartorius)	0.01-0.015	Pitched blade (x1)	63/31	Single use vessels	(Rafiq et al., 2017)
BIOSTAT® STR Plus (Sartorius)	12.5-50	3-blade seg. or Rushton turbine (x2)	666/370	Single use bags	(Schirmaier et al., 2014)
BIOSTAT® B Plus (Sartorius)	0.6-5 [I]	45°-pitch wide blade (x3)	730/340	Autoclavable glass vessel and single use vessel available	(Lam et al., 2017; Rafiq et al., 2013)
BIOSTAT® B-DCU II (Sartorius)	0.15-0.5[I]	6-blade disk impeller (x2)	340/170	Autoclavable glass vessel	(Chen et al., 2015; Goh et al., 2013; Hupfeld et al., 2014)
BIOSTAT® Qplus (Sartorius)	0.4-1	3 blade seg. (x1)	340/170	Autoclavable glass vessel	(Cunha et al., 2015)
Celligen® 310 (New Brunswick)	0.75-1.75	45°-pitch blade (x3)	580/230	Autoclavable glass vessel	(Mizukami et al., 2016)
DASbox ® (Eppendorf)	0.32-1.25	Pitched-blade (x1)	140/105	Both single use and glass vessels available	(Dos Santos et al., 2014)
Mobius® (Merck)	3 50	3 blade marine (x1) 4-pitched blade (x1)	249/137	Single use	(Lawson et al., 2017)
UniVessel® (Sartorius)	0.6 to 2	3 blade seg. (x1)	242/130	Single Use	(Jossen et al., 2014; Schirmaier et al., 2014)

[I] Glass vessels with different working volumes (UniVessel®) are available for the BIOSTAT® B Plus/BIOSTAT® B-DCU II (0.5, 1, 2, 5 and 10 L).

[II] Data obtained from the manufacturers' catalogues.

4.7. **Microcarriers**

The commercial microcarriers currently available exhibit very different properties which has been shown to impact cell growth. They can differ with respect to their diameter, matrix, density, available surface area, coating, charge, shape and porosity. Microcarrier diameters often range from

100 up to 300 µm, whilst the microcarrier material varies from polystyrene, porcine gelatine, dextran and PVA. Depending on the microcarrier, some have different surface coatings which can range from porcine collagen to DEAE, CellBIND®, silica glass and cationic trimethyl ammonium among others (Qasim A. Rafiq et al., 2016). In terms of charge, microcarriers are typically either positively or neutrally-charged since mammalian cells have negative charge. One disadvantage of using charged microcarriers is related to the non-specific binding between serum proteins from the culture medium and the microcarriers (Mukhopadhyay et al., 1993). This phenomenon may have impact both in cell based and cell-free products, suggesting the need of adopting neutrally charged microcarriers to avoid the presence of contaminant proteins in the first steps of the downstream processing.

With respect to porosity, microcarriers can be either non-porous, micro or macro-porous. In terms of carrier porosity, non-porous microcarriers only allow cell attachment and growth on the external surface area of the carrier while macroporous microcarriers enable cells to bind to the internal pores of the microcarrier. The key advantage of using macroporous microcarriers is the availability of increase surface area per microcarrier. However, the harvesting procedure is more complex, especially when attempting to remove the cells on the inner surfaces of the microcarriers (Ma and Su, 2013). Moreover, maintaining homogeneity during the cell inoculation phase can be challenging, particularly with respect to cell distribution. Furthermore, the phenomenon of bead-to-bead transfer, which has been proven for non-porous microcarriers (Rafiq et al., 2018) is unlikely to be possible with macroporous carriers given that the cells will attach and grow inside of the macroporous carrier. Non-porous microcarriers pose fewer problems when harvesting; however, with the cells being attached only to the external surface of the microcarrier, cells are more exposed to detachment arising from bubbles when sparging is employed (as discussed earlier) and fluid dynamic stresses (Heathman et al., 2018). When operating at higher cell densities, there is the challenge of meeting high oxygen demand using other methods than sparging, given that sparging has detrimental effect on cell growth (Heathman et al., 2018). It was proposed a mechanism to systematically screen microcarriers in a high-throughput manner in both static culture systems as well as agitated vessels (Rafiq et al., 2016). This work showed that the microcarriers which led to the highest cell yields in microwells also did so in the stirred bioreactor. Irrespective of cell type, it will be critical that such studies are undertaken to ascertain the optimal microcarrier for cell growth and/or functionality.

In addition to porosity, another feature of newly developed microcarriers is the ability to develop microcarriers that are biodegradable. Biodegradable microcarriers may be preferential for certain clinical applications if the cells being cultured will form part of a wider scaffold construct (Koh et al., 2020) or if there is a concern about the risk of administering a cell product with microcarrier fragments (Willerth and Sakiyama-Elbert, 2019). Furthermore, other microcarriers have been developed, including by Corning® and others, which allow for microcarriers to dissolve upon shifts in temperature (Kalra et al., 2019), i.e. thermoresponsive microcarriers. A common polymer used is poly (N-isopropylacrylamide), also referred to is PNIPAM where it was demonstrated by Yuan *et al.* 2018 that cells could be expanded on microcarriers developed with this type of polymer (Yuan et al., 2018). In addition to thermoresponsive microcarriers, alginate-based microcarriers have also been designed which are degraded through the cleavage of chemical crosslinkages. Li *et al.* 2016 demonstrated that alginate/PEG-based microcarriers could be used for the expansion of human umbilical cord blood-derived MSCs, and that by changing the type and concentration of the chemical reductant, the microcarriers could degrade and the cells could be harvested microcarrier-free, without the need for filtration (Li et al., 2016). This approach provides potentially significant advantages with respect to the downstream processing, effectively removing the need for

microcarrier separation. However, further studies need to be conducted to investigate the impact of these temperature changes and changes in chemical reductants on the quality of the cells.

4.8. Bead-to-bead transfer

During the hMSC expansion process on microcarriers in a STR, confluency becomes a major bottleneck with a lack of available surface area to facilitate cell growth. However, it has recently been demonstrated both qualitatively and quantitatively, that hMSCs are able to transfer from a confluent microcarrier to an empty microcarrier in a process referred to in the field as 'bead-to-bead transfer' (Rafiq et al., 2018). One of the key advantages of bead-to-bead transfer is that this allows the available surface area to be increased during the course of the culture through the addition of fresh microcarriers, thereby avoiding the need to harvest prematurely to increase surface area and minimises the need for extensive pre-culture steps in tissue flasks that are poorly controlled and require human handling (Leber et al., 2017). This phenomena happens when cells bridge between microcarriers or when cells attach to new microcarriers (Rafiq et al., 2018). This phenomenon has been demonstrated with hMSCs (Qasim A. Rafiq et al., 2016; Rafiq et al., 2018) and other cell types including Vero (Wang and Ouyang, 1999) and CHO cells (Ohlson et al., 1994). The phenomenon has also been demonstrated for immortalised hMSC cell lines (Leber et al., 2017) and across multiple medium conditions including FBS, hPL and serum-free media (Heathman et al., 2016; Heathman et al., 2015). Bead-to-bead transfer was shown to improve the cell growth rate and cell yield compared to standard inoculation methods and was demonstrated for multiple BM-hMSC donors. However, increases in microcarrier concentration require slightly higher agitator speeds to ensure that suspension is maintained (Heathman et al., 2018; Nienow, 2006). Moreover, an increase in the microcarrier concentration will increase the collisions that occur between beads; added to the additional increase in agitation required to suspend these beads, there is potential for these collisions to adversely impact hMSC culture viability. This possibility should be considered when facilitating the culture with the addition of fresh microcarriers.

5. Key challenges in hMSC-microcarrier culture

In addition to monitoring, controlling and optimising the key culture parameters mentioned above, it is critical that key challenges currently impact hMSC-microcarrier culture are addressed to increase final cell yield. This involves identifying aspects of the process which contribute to cell loss and developing strategies to mitigate these effects. Whilst the aforementioned parameters have a direct impact on the process outcome, there are critical challenges that require significant R&D activity to develop an optimised production process.

5.1. Attachment Phase

The attachment and harvesting steps are key phases in the hMSC production process (Nienow et al., 2014). Given the anchorage-dependent nature of hMSCs, they require a surface in order to attach and proliferate. The lack of such a substrate results in cell death through anoikis (Gilmore, 2005). Whilst this is relatively straightforward for monolayer culture where the seeded cells come into contact with the plastic surface through the force of gravity, microcarrier cultures usually involve significantly larger working volumes and cell contact with microcarriers is less predictable.

To facilitate cell attachment to the microcarriers, several strategies have been adopted: (1) a static attachment phase, effectively allowing the cells to descend via gravity (Hervy et al., 2014; Qasim A. Rafiq et al., 2016; Rafiq et al., 2013; Schirmaier et al., 2014), (2) static followed by a period of agitation (typically at a lower speed compared to the one used during the expansion) (Carmelo et al., 2015; Caruso et al., 2014; Di Naro et al., 2001; Dos Santos et al., 2014; Lam et al., 2017; Santhagunam et al., 2014; Santos et al., 2011; Sun et al., 2010; Takahashi et al., 2017; Tozetti et al., 2017) or (3) continuous agitation (de Soure et al., 2017; Mizukami et al., 2016; Petry et al., 2016; Shekaran et al., 2015; Tan et al., 2015). The rationale behind using the static attachment strategy, is to promote contact between cell and microcarriers. It is thought that intermittent agitation and static cycles promote a combination of mixing and sufficient contact time. Continuous agitation approaches involve no static periods and the cells attach to the microcarriers during the course of the culture. There appears to be no single 'optimal' approach in the literature, with many groups adopting their own strategy. However there is recognition that a compromise is required to ensure sufficient cell-microcarrier contact whilst avoiding undesired microcarrier aggregation (Goh et al., 2013; Petry et al., 2016). Adhesion efficiency reported can be as low as 22-23% (Santos et al., 2011) and up to 90% (Carmelo et al., 2015; Goh et al., 2013; Mizukami et al., 2016; Tan et al., 2015). This is one of sensitive aspects of microcarrier-based MSC expansion because low levels of adherence will generate lag phases of several days and not reaching the desired cell yield (Goh et al., 2013; Mizukami et al., 2016; Santos et al., 2011). Although attachment is a key parameter to monitor, its optimisation depends on multiple factors such as microcarrier choice, agitation speed and working culture volume.

5.2. **Cell harvesting**

Isolating a single cell suspension after expansion whilst maintaining cell quality presents a key process step and one that is specific to the CGT industry. Traditional bioprocessing involves the manufacture of products which are secreted by the cells; hence preservation of the cell was not essential. For CGTs, the cell forms the basis of the product and there is a need to ensure cell quality is not deleteriously impacted by the processing conditions.

Nienow et al. (2014) outlined a scalable method for harvesting hMSCs from a STR-microcarrier culture *in situ*. This method involved a two-step process: (1) detachment of the cells from the microcarriers; and (2) separation of the cells from the microcarriers. The detachment process required both an enzymatic solution and an increase in the agitation speed during the harvesting period. The separation of the cells from the microcarriers involved a vacuum filtration process (Heathman et al., 2015; Nienow et al., 2014). Loss of cell viability is one of the concerns while trying to scale up harvesting techniques. However, reported harvesting efficiencies of viable cells using this method has been generally high (>80%) (Nienow et al., 2014). The same protocol was included while establishing a whole manufacturing processing (expansion, harvest and cryopreservation) (Heathman et al., 2015). It was also reported that there was no impact on cell apoptosis, metabolic activity and cell adhesion while using a tangential flow filtration module (Cunha et al., 2015). However, the harvesting process, and downstream processing more generally, is an area that requires significant optimisation. In particular, clumping poses a problem for dettachment harvesting of high-cell density cultures (Borys and Papoutsakis, 1992; Rafiq et al., 2017); and especially an alternative for vacuum filtration is required as this is not a scalable method for separation.

5.3. **Feeding Strategy**

An advantage of using a STR platform is the ability to operate the culture under different modes of operation including (1) batch, (2) perfusion (3) fed-batch and (4) draw-fill. Whilst batch culture involves the addition of cells and culture medium at the beginning of the process with no further additions and perfusion culture involves the addition and removal of culture medium at a specified rate, in fed-batch, medium is added but not removed. Though all of these are common in bioprocessing in general, draw-fill culture is the primary mode of operation for the majority of hMSC-microcarrier studies in the literature and is characterised by the withdrawal and addition of medium during the course of the expansion process. Studies adopting this approach often involve a 25% - 50% medium exchange every 2 or 3 days (Dos Santos et al., 2014; Heathman et al., 2016; Heathman et al., 2015; Hervy et al., 2014; Kaiser et al., 2013; Lam et al., 2017; Nienow et al., 2014; Rafiq et al., 2013; Santos et al., 2011), with some studies opting to use a medium highly concentrated in glucose to optimise cell productivity. This strategy reduces medium usage (Chen et al., 2015; Dos Santos et al., 2014; Lam et al., 2017; Petry et al., 2016), avoiding glucose depletion in the medium below an established threshold (Chen et al., 2015; Dos Santos et al., 2014; Lam et al., 2017; Petry et al., 2016). It was reported that using highly concentrated glucose in the medium resulted in more efficient medium utilisation when compared to a standard draw-fill culture process (1.7 ± 0.1 *versus* 5.3 ± 0.3 mL of medium/ 10^6 cells at the end of the culture) (Lam et al., 2017). Although not reported in the latter study, osmolarity must also be considered as the impact of high metabolite concentrations may have an impact on cell quality (as it does in free suspension culture (Nienow, 2006)). Although most of the studies use draw-fill as feeding strategy, due to the lower medium usage efficiency, this option is it not likely to be taken forward when scaling up the production. As highlighted by Lam et al 2017, choosing fed-batch as a feeding strategy will result in 70% of medium usage reduction leading to a decrease in the production costs. Additionally to production cost reduction achieved when operating in fed-batch mode, the authors also highlighted a reduction in the culture system manipulations, an key advantage when considering the commercial scale (Dos Santos et al., 2014).

With respect to cell yield, no difference has been reported between a 25% medium exchange daily and exchange every other day (Dos Santos et al., 2014). In the same study, it was reported that a run using a perfusion process (with a perfusion rate of 100 mL/day using XF medium) yielded 5.0 x 10^5 cells/mL after 11 days. While operating in perfusion mode may result in higher cell densities, the increase in medium utilisation may results in higher costs. Also, although waste product removal would be better than in batch cultures, the continuous removal of medium may dilute key growth factors such as small molecules and cytokines important for cell growth and functionality; and consideration of osmolarity is important to ensure that this remains in an acceptable range to facilitate effective cell growth.

5.4. **Aggregate formation**

Once attached, hMSCs will continue to proliferate on the surface of the microcarriers until all the available area is covered by cells. During the course of the culture, cells continue to proliferate and microcarriers collide which tends to result in cell-microcarrier aggregates (clumping) (Schop et al., 2010). Clumping is an undesirable as these cell aggregates increase the heterogeneity of the culture, resulting in non-reproducible cell counts and may also cause cell death when the cell aggregates impede mass transfer to or from the cells (Ferrari et al., 2012; Rafiq et al., 2017). Even if the product of interest is the secretome, cell death is undesirable as cell death pathways might trigger undesired microvesicle production. To prevent aggregate formation two strategies can be

used: (1) modulating agitation during the culture (Jossen et al., 2016); or (2) addition of fresh microcarriers to enable the transfer of cells from full microcarriers to empty ones (also known has bead-to-bead transfer) (Heathman et al., 2016; Heathman et al., 2015; Leber et al., 2017; Ohlson et al., 1994; Rafiq et al., 2016; Rafiq et al., 2018; Wang and Ouyang, 1999).

Agitation intensity is one of the most critical parameters to be considered. As already discussed, and expanded upon below, the agitation intensity should ensure that all microcarriers are suspended but at levels just above that cell aggregation may occur. On the other hand, others have reported that high levels of agitation have caused detrimental effects on growth kinetics (Jossen et al., 2016; Yuan et al., 2014), cell viability (Jossen et al., 2016; Yi et al., 2010), differentiation ability (Yourek et al., 2010) and clonogenic ability (Lam et al., 2017). However, the phenomenon of cell 'damage' and these related effects being linked inexplicably to agitation rates is contentious and often unproven. Indeed, there were similar concerns expressed in the early days of mammalian cell culture which were reported to be 'shear sensitive' despite much work to show that cells are less sensitive to fluid dynamic stresses generated by agitation than originally thought (Nienow, 2006). The agitation speeds have been as low as 30 rpm in spinner flasks (Chen et al., 2015; Heathman et al., 2016; Heathman et al., 2015) up to 60-80 rpm (Chen et al., 2015) or even 100-140 rpm in larger bioreactors (Schirmaier et al., 2014). In general, the reason for the particular choice has not been given explicitly.

Agitation speed on its own cannot be used to compare bioreactors. Usually impellers have different relative sizes compared to the dimension of the bioreactor and different shapes; and the geometry of bioreactors generally varies too. A better option for comparing between different bioreactors is to use the mean specific energy dissipation rate (W/kg or W/m^3) into the medium which is exactly equivalent to the specific power imparted by the impeller with the same units (P/M or P/V – in a water-like medium, numerically the specific power,P/V W/m^3 = 1000 P/M W/kg). (Table 3). The mean specific energy dissipation rate, $\bar{\varepsilon}_T$ (W/kg) at the impeller speed N (rev/s) can be calculated using Equation 1:

$$\bar{\varepsilon}_T = \left(\frac{P}{M}\right) = \frac{P_0 \rho_L N^3 D^5}{M} \qquad \text{Equation 1}$$

where Po represents the impeller power number (dimensionless) which is specific for each impeller, D, it's diameter (m), ρ_L the density of the medium (kg/m^3) and M the mass of medium and microcarriers inside of the vessel. The maximum local value of ε_T which also varies with impeller type is generally considered to be the parameter which most closely relates to the possibility that fluid dynamic stresses cause a change in culture performance or cell quality (Nienow et al., 2016)

In Table 3, power per unit volumes (P/V W/m^3) used in successful MSC cultivations are given calculated using values from literature. It is clear that though P/V is often used to give similar culture performance in different stirred tank bioreactors and also used as scalability criteria, here very different values are seen. This difference arises because as fundamental mixing studies (Nienow, 1997) have shown, depending on the precise geometry of impeller and reactor, the P/V for the same type and concentration of particles can vary by as much as an order of magnitude. Since microcarrier suspension is such an important basic need in microcarrier culture, it is much more advantageous to use N_{JS}, the minimum speed for particle suspension as the basic criterion for scale-up (Nienow et al., 2016). Small increases in speed to mitigate clumping can then be added as required. For precisely geometrically-similar bioreactors and impellers, scale-up at equal P/V should ensure good

suspension (Nienow, 1997). Unfortunately, as already suggested, geometric similarity is extremely rare and bioreactors configurations, especially in single use bioreactors, are also rarely of the type for which accurate literature data are available to enable N_{JS} to be calculated. The use of CFD may provide a solution to this dilemma (Jossen et al., 2014; Schirmaier et al., 2014).

Table 3- Summary table with power per unit volumes calculated from studies focusing on hMSC bioreactor-based expansion using microcarriers.

Bioreactor	N (rpm)	Po[I,II]	Working V. (L)	P/V (W/m^3)	Ref.
Ambr® 15	300	2.1	0.015	2.81	(Rafiq et al., 2017)
Ambr® 15	450	2.1	0.015	9.49	(Rafiq et al., 2017)
Spinner Flask	40	1	0.1	1.49	(Lam et al., 2017)
Spinner Flask	60	1	0.1	5.02	(Schirmaier et al., 2014)
Spinner Flask	50	1	0.1	2.91	(Mizukami et al., 2016)
DASbox ®	60	1.5	0.8	1.97	(Dos Santos et al., 2014)
BIOSTAT® B Plus	75	1.5	2.5	0.94	(Rafiq et al., 2013)
Mobius®	75-100	1.3	50	1.92 to 4.55	(Lawson et al., 2017)

[I] Power numbers obtained from published literature (Nienow et al., 2016).

[II] Data obtained from the manufacturers' catalogues.

5.5. Quality Control and Analytics

To ensure that the CGT product meets the quality requirements established by regulatory agencies, an extensive set of quality control tests is routinely performed (Lechanteur et al., 2016; Viganò et al., 2018). Together with potency assays (section 3.1), a typical battery of QC tests for CGT can include purity, viability, genetic stability and, immunophenotype characterization (Sun et al., 2016). During process development, it is critical to identify the appropriate QC tests to be performed at each stage.

CGT manufacturing processes often start with an isolation step (section 4.1), where a sub-population of cells is separated from a heterogenous pool of cells or tissue (Jin et al., 2013; Simões et al., 2013, Fazzina et al., 2015; Mori et al., 2015; Timmins et al., 2012, Secco et al., 2008). Given that isolation protocols usually rely on enzymes and/or chemicals as separation agents, viability assessment is especially critical at this point (Timmins et al., 2012). Additionally, at the end of the isolation process, an immunophenotypic characterisation step (Jin et al., 2013; Simões et al., 2013,

Fazzina et al., 2015; Mori et al., 2015; Timmins et al., 20120, Secco et al., 2008) is usually performed to ensure the isolated cells are indeed hMSCs (Dominici et al., 2006)

As cell growth constitutes the primary focus of an expansion step, it is key to assess whether genetic mutations, such as chromosomal rearrangements, have occurred as a consequence of cell division (Neri, 2019). A common approach is to perform karyotype analysis (Jones et al., 2013; Zhao et al., 2015). Given that the number of divisions a human cell undergoes has a maximum (also known as the Hayflick limit (Shay and Wright, 2000)), it is important to include senescence assays after expansion. Some of these assays include telomere length assessment (Dos Santos et al., 2011; Dos Santos et al., 2014) and apoptosis assays, such as Annexin-V (Schirmaier et al., 2014) or Caspase-3 (Sousa et al., 2015). While a telomere length assay evaluates the extent to which the proliferative potential has been affected during the expansion cycle, Annexin-V and Caspase assays quantify the cellular fraction that show early signs of replicative senescence.

Purity assays should be performed during the harvesting procedure to ensure that the final CGT product does not contain any microcarrier particles or fragments. Such assays are especially important when using glass, plastic, porcine or any other inert substance that is commonly used for microcarrier manufacturing instead of biodegradable microcarriers (Lam et al., 2017). To study whether microcarriers are damaged during culture, either due to turbulence or due to microcarrier-microcarrier collisions, Hewitt et al., 2011 conducted a study using Cytodex-3 microcarriers suspended at the Njs (section 5.4) for 13 days. The authors reported no changes in microcarrier morphology or size distribution, or any signs of stress such as lines or fractures at the microcarrier surface level. This study suggests that Cytodex-3 microcarriers are unlikely to be damaged if suspended at the Njs. To evaluate whether there are microcarrier particles released during the manufacturing process, a similar study needs to be performed on a case-by-case basis as microcarriers have different structural properties and impeller configuration may change between different bioreactors.

Due to the nature of their characteristics, hMSC exosome products have different QC assays when compared to hMSC cell based products (Baldari et al., 2017; Rohde et al., 2019). Although cell counting, viability determination and immunophenotype are usually part of the QC panel of an exosome product, additional tests need to be included. These assays include determination of exosome quantity, size and their surface marker profile (usually performed using Western blots as opposed to flow cytometry routinely used in hMSC cell based products (Kowal et al., 2017; Ramirez et al., 2018; Shao et al., 2018). Although the manufacturing process uses hMSCs, sterility, mycoplasma or endotoxin contamination assays are performed on the exosomes as they constitute the final product (Rohde et al., 2019).

6. Future Perspective and Conclusion

From a clinical perspective, there is an increasing focus on gene editing hMSCs or using hMSCs for the production of exosomes. These therapeutic modalities will certainly impact the manufacturing process and as such, impact R&D and process development activity. As an increasing number of clinical trials using hMSCs reaches later stages of development, there is an urgent need to optimise the bioprocesses to increase cell yield and decrease production costs. Many parameters affect cell yield and they need to be carefully modulated to optimize the concentration of the desired product. The current manufacturing processes have been optimized for cell production where the product is the cell itself, but we anticipate a new wave of studies investigating the development of bioprocesses for the scalable production of hMSC-derived exosomes and gene-

modified hMSCs. A key question moving forward is whether the current hMSC-microcarrier bioprocesses are appropriate for exosome production or gene-modified hMSC manufacture. Key questions need to be addressed such as whether the concentration of exosomes (and their identity) changes with an increase in cell concentration during the process. It is likely however that the production of specific cell products will require different bioprocessing conditions to facilitate optimal manufacture, and therefore, it is now imperative more than ever that we monitor and control the key bioprocess parameters during hMSC-microcarrier culture and address key manufacturing bottlenecks.

Table 4-Process parameters modulation summary and their impact in cell yield and potency for the manufacturing of hMSC.

Parameter	Set by Default	Modulation	Impact on Cell Yield and Characterization	Impact on Potency	Ref
Temperature	37 °C	Decreasing Temperature	Not reported	Not reported	(Dos Santos et al., 2014; Kumar et al., 2008; Rafiq et al., 2013)
pH	7.1-7.8	Increasing pH Decreasing pH	Increased attachment efficiency Decreased attachment efficiency	Not reported	(Caruso et al., 2014)
Oxygen and Aeration	20 to 45% of air Saturation	Decreasing O_2 Usually air is introduced in the headspace	Hypoxia might increase proliferation and a more efficient cellular metabolism in monolayer. Impact on suspension-based systems is not clarified. Sparging air directly into the culture medium might affect cell attachment	Might increase clonogenic ability	(Dos Santos et al., 2014, 2010; Estrada et al., 2012; Heathman et al., 2018)
MSC Source	BM, AT, UC Others	Different cell sources	Perinatal sources show higher proliferation ability	Perinatal sources may have improved immunomodulatory properties	(Bárcia et al., 2015; de Witte et al., 2017; Li, 2014; Simões et al., 2013)
Medium use	DMEM, α-MEM or SF/XF	Different medium formulations	Higher proliferation ability of SF/XF options when compared with FBS-supplemented medium	Not reported	(Martin et al., 2015; Rafiq et al., 2017; Simões et al., 2013)
Serum use	FBS (10-20%), hSerum, hPL	Using different medium supplements	Higher proliferation ability while using hPL compared to FBS	hPL might reduce immunossupressive ability of MSC	(Heathman et al., 2016; Oikonomopoulos et al., 2015)
Pre-Expansion	P2-P8	High passage Low passage	Senescence and slower growth kinetics at high passage	High level of doublings might reduce immunomodulatory and clonogenic ability	(de Witte et al., 2017; Simões et al., 2013)
Microcarrier Type	Different types of microcarriers	Different types of microcarriers	Microcarriers with different properties yield different attachment efficiencies, cell concentrations, cell viability, metabolic profile and differentiation ability.	Not reported	(Lam et al., 2017; Lin et al., 2016; Petry et al., 2016; Qasim A. Rafiq et al., 2016; Takahashi et al., 2017; Timmins et al., 2012)

Attachment phase	Agitated, rested or both	Type of attachment phase, duration	Agitation and rested cycle improve cell attachment to the microcarrier and maximizes viability compared to continuous agitation	Not reported	(Rafiq et al., 2017; Yuan et al., 2014)
Agitation	Related to microcarrier suspension, impeller type and scale	Minimum N_{JS} Higher N to control aggregate formation ↓agitation rate	Less formation of cell aggregates. Optimized agitation rate impacts on cell yield, growth kinetics and metabolic profile	Specific power relates to hydrodynamic stresses which might impact on differentiation of MSC	(Jossen et al., 2016; Rafiq et al., 2017; Takahashi et al., 2017; Yi et al., 2010; Yuan et al., 2014)
Feeding Strategy	Fed-Batch w/ 25% to 50% med. exchange every 2-3 days	Using different feed compositions or perfusion	Highly concentrated glucose feeds and perfusion system might increase cell concentration.	Not reported	(Dos Santos et al., 2014; Lam et al., 2017; Petry et al., 2016)

Captions

Figure 1- Schematic representation of the challenges of developing hMSC-based therapies using microcarrier-based bioprocesses under suspension conditions.

Figure 2- Graphical representation of the maximum cell yields reported in literature together with working volume in the studies.

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