Delineation of optimal culture conditions for the production of therapeutic human mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in static cultures

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Preface

The work presented in this thesis was performed at the Stem Cell Engineering Research Group (SCERG) at TagusPark of Instituto superior Técnico (Lisbon, Portugal), during the period March-September 2021, under the supervision of Prof. Ana Margarida Pires Fernandes-Platzgummer and co-supervision of Doctor Raquel Medina dos Santos Cunha.

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

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Abstract

Mesenchymal stromal cells (MSCs) have been studied for therapeutic applications due to their promising properties. In the last years, MSC therapeutic effects have been associated to the release of extracellular vesicles (EVs) as paracrine signaling. MSC-EVs therapeutic potential has already been reported as beneficial for different diseases and seem to recapitulate the therapeutic effects of MSCs, creating expectations to a promising, safe, and effective cell-free-based therapy. However, a considerable amount of EVs is needed for therapeutic applications and thus, cell culture processes need optimization to ensure high productivity, combined with quality, consistency, reproducibility, and safety.

In this manner, this project involves the study of different cell culture parameters (MSC source, time points for culture medium exchange and EV collection, temperature and oxygen concentration) to obtain an optimized human MSC EVs-based product. Overall, the MSC source and the medium exchange seemed not to imply significant variability in EV productivity, and the time point after MSC expansion and medium conditioning seemed the best one for EV collection. Regarding the removal of culture medium particles that could have contaminated the MSC-EV samples, a filtration with 0.1 µm filter seemed inefficient. The temperature of 33ºC applied during conditioning medium seemed to increase the EV productivity, contrarily to hypoxia, but further studies are necessary.

In sum, besides the necessary further studies, the present work provides insights regarding the influence of different cell culture parameters in MSC-EV production that can contribute to reach in the future the best quality/potency and productivity of MSC EV-based products.

Keywords: extracellular vesicles (EVs); mesenchymal stromal cells; cell culture parameters; EV productivity.

Resumo

Células Mesenquimais Estromais (MSCs) têm sido estudadas para aplicações terapêuticas pelas suas propriedades promissoras. Recentemente, efeitos terapêuticos das MSCs foram associados à secreção de vesículas extracelulares (EVs) como sinalização parácrina. O potencial terapêutico das MSC-EVs já foi descrito como benéfico para diversas doenças e parece recapitular os efeitos terapêuticos das MSCs, criando expectativas para uma terapia sem células promissora, segura e eficaz. No entanto, uma quantidade considerável de EVs é necessária para ser aplicada em terapia, sendo necessário otimizar os parâmetros de cultura celular para garantir alta produtividade, simultaneamente com qualidade, consistência, reprodutibilidade e segurança.

Assim, este projeto envolve o estudo de diferentes parâmetros de cultura celular (fonte de MSC, instante para mudança de meio e recolha de EVs, temperatura e oxigénio) para obter um produto MSC-EVs otimizado baseado na produtividade. A fonte de MSCs e mudança de meio não implicaram variabilidade na produtividade de EVs, e o momento após a expansão de MSC e condicionamento do meio pareceu melhor para recolher EVs. Em relação a remover partículas do meio de cultura que poderiam ter contaminado amostras de MSC-EV, filtração com filtro 0,1 µm pareceu ineficiente. A temperatura de 33ºC aplicada durante o condicionamento do meio pareceu aumentar a produtividade de EVs, ao contrário da hipoxia, mas mais estudos são necessários.

Em suma, apesar dos estudos futuros necessários, este trabalho fornece elucidações sobre a influência de diferentes parâmetros de cultura celular na produção de MSC-EV que podem contribuir para alcançar, no futuro, a melhor qualidade/potência e produtividade dos produtos EV.

Palavras-chave: vesiculas extracelulares (EVs)**;** células mesenquimais estromais; parâmetros de cultura celular; produtividade de EVs**.**

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Abbreviations

Adipose tissue (**AT**)
Atomic force microscopy (**AFM**)
Bone marrow (**BM**)
Bovine serum albumin (**BSA**)
Clinical Good Manufacturing Practice (**cGMP**)
Conditioned Medium (**CM**)
Endosomal sorting complex required for transport (**ESCRT**)
Extracellular Vesicles (**EVs**)
Fetal bovine serum (**FBS**)
Fourier-transform infrared spectroscopy (**FTIR**)
Good Manufacturing Practices (**GMP**)
Graft-versus-host disease (**GvHD**)
Human platelet lysate (**hPL**)
International Society for Cellular Therapy (**ISCT**)
Medium exchange (**ME**)
Mesenchymal stromal cells (**MSCs**)
MicroRNAs (**miRNAs**)
Mononuclear cells (**MNC**)
Nanoparticle tracking analysis (**NTA**)
Paraformaldehyde (**PFA**)
Polyethylene glycol (**PEG**)
Scanning electron microscope (**SEM**)
StemPro® MSC SFM XenoFree medium (**SP**)
StemPro® MSC SFM XenoFree filtered medium (**SPf**)
The International Society for Extracellular Vesicles (**ISEV**)
Transmission electron microscopy (**TEM**)
Tris-buffered saline (**TBS**)
Umbilical cord matrix (**UCM**)
Whole cell lysates (**WCL**)
Working reagent (**WR**)

1. Introduction

1.1. Human Mesenchymal stromal cells

1.1.1. Designation and Characterization

Biologic and clinical interest in Mesenchymal stromal cells (MSCs) has risen over the last decades due to their promising potential for several therapeutic applications, making them one of the most employed cell types under investigation as an experimental cell-based therapy for treating human diseases. This attraction to the field undoubtedly accelerated the scientific discovery and the development of novel cellular therapies. However, this rising interest and the growth of MSCs published studies have also generated many discord and inconsistencies (Dominici et al. 2006).

One of the inconsistencies was about the designation of these cells. Until the early twenty-one century, “Mesenchymal stem cell” was the designation commonly applied to the plastic-adherent cells isolated from bone marrow (BM), adipose and other tissues, with multipotent differentiation capacity *in vitro.* However, some pre-clinical or clinical studies of Mesenchymal stem cells from the latter half of the 1990s decade lacked a reference to any stem cell identity. Consequently, the International Society for Cellular Therapy (ISCT) concluded in the year 2000 that convincing data to support the ‘stemness’ of the unfractionated plastic adherent cells were missing. Therefore, one of the needs was to clarify the nomenclature for these important cells and to create a scientifically accurate standardized terminology that allows an easy exchange of knowledge among biomedical investigators and transmission of information to the general public without giving unrealistic expectations (Horwitz et al. 2005). In 2005, the ISCT MSC committee proposed that the plastic-adherent cells should be designated as multipotent mesenchymal stromal cells, indicating a bulk population with notable secretory, immunomodulatory and homing properties. The term mesenchymal stem cell should be reserved for a subset of these (or other) cells that demonstrate stem cell activity by clearly stated criteria, referring to a stem cell population with demonstrable progenitor cell functionality of self-renewal and differentiation into specific, multiple cell types *in vivo* (Horwitz et al. 2005; Dominici et al. 2006). The goal was to suggest this terminology as the most scientifically accurate descriptor to avoid the implication of unproven biological or therapeutic potential.

Furthermore, it became clear that distinct laboratories were using different ways to isolate, expand and characterize MSCs, leading to the proposal by ISCT committee, in 2006, of the minimal criteria to define human multipotent MSCs (Dominici et al. 2006). This intended to address the inability to compare and contrast the resulting cells from different studies and the reported biologic properties as well as experimental outcomes, especially in the context of cell therapy, which is likely to hinder progress in the field. Therefore, the set of standards to define human MSCs for both laboratory-based scientific investigations, pre-clinical and clinical studies include: firstly, plastic adherence when maintained in standard culture conditions using tissue culture flasks; second, the expression of specific surface antigens CD73, CD90 and CD105, by more than 95% of the MSC population, while lacking the expression of hematopoietic and endothelial surface markers CD11b, CD14, CD19, CD34, CD45,

CD79a and HLA-DR (less than 2% of MSC population can be positive); and thirdly, the capability of *in vitro* differentiation into adipocyte, chondrocyte and osteoblast lineages (mesoderm differentiation potential), to demonstrate their multipotency (Dominici et al. 2006).

MSCs may have different morphology according to culture conditions. However, using standard culture conditions, MSCs typically have a spindle-shaped, triangular shaped and fibroblastic like morphology (Liu et al. 2016).

1.1.2. MSCs properties and Clinical applications

MSCs are normally isolated from human BM and adipose tissue (AT) but have also been identified in other tissues, including perinatal tissues, such as the umbilical cord, the membrane surrounding the embryo called the amnion, the placenta and Wharton's jelly (a gelatinous substance within the umbilical cord). This cells were also found in menstrual blood and human dental pulp (Sipp et al. 2018). MSCs present multilineage differentiation ability, which allows the replacement of cells that undergo apoptosis, being important to maintain specific tissues, and assure support in case of small focal repair needs. MSCs also have trophic capacity described by the secretion of certain growth factors and cytokines important in tissues response to injury and contributing to the reparative effects (Dennis and Caplan 2004). Furthermore, MSCs have immunoregulatory functions, influencing various components of the innate immune system and vice versa (Le Blanc and Mougiakakos 2012), and also homing properties (Kallmeyer and Pepper 2015). In this manner, MSCs possess several characteristics that make them suitable for cell therapy applications, including the ability to prevent apoptosis, to promote proliferation, migration and angiogenesis, capacity to suppress fibrosis and scar formation, and supportive function (Lin et al. 2011). Therefore, these cells have been widely employed in clinical trials for treating numerous human diseases in the last decades.

In the case of *in vivo* administration of MSCs, they are reportedly capable to migrate to and accumulate at sites of damaged tissue, such as in case of musculoskeletal injury (Fong et al. 2011), brain lesion (Iihoshi et al. 2004) and cardiac infarct (Bittira et al. 2003; Nagaya et al. 2004), and seem to contribute to the regeneration of tissues and reinforcement of the inherent reparative capacity of the body. Accomplished clinical trials testing MSCs with encouraging results include children with osteogenesis imperfecta (Horwitz et al. 1999; Horwitz et al. 2002), patients with metachromatic leukodystrophy or Hurler syndrome (Koç et al. 2002), and patients with myocardial infarction (Wollert et al. 2004). Similarly, MSC were tested in animal models of different diseases with encouraging results, such as in promoting fracture-healing (Tseng et al. 2008). Moreover, MSCs' ability to modulate immune responses makes them an attractive tool for cellular therapy in allo-immunity, autoimmunity and inflammation. Therefore, they were also tested in clinical trials for acute graft-versus-host disease (GvHD) (Le Blanc et al. 2008; Prasad et al. 2011), Crohn's disease (García-Olmo et al. 2005; Duijvestein et al. 2010) and amyotrophic lateral sclerosis (Mazzini et al. 2010), demonstrating promising results.

The therapies based on MSCs started to focus on their capacity to differentiate, engraft and replace cells in the damaged and diseased tissues by the generation of new healthy cells. However, the differentiation and transdifferentiation mechanisms started to be considered unlikely. Indeed, the probability of MSC engraftment and differentiation upon administration is low and, although some cells home to the injured target tissue, several studies already demonstrated that MSCs get entrapped in the

capillaries of the lung when intravenously administered and are largely cleared. Furthermore, it was also observed that the treatment is due to mechanisms other than the migration of transplanted MSCs to the injured tissue (Zhang et al. 2016; Phinney et al. 2017). Multiple studies pointed the secretion of paracrine bioactive molecules as an alternative and more acceptable mechanism by which MSCs stimulate the host cells and largely exert their therapeutic effects, such as tissue repair.

1.1.3. Paracrine Activity

MSCs, like stem cells in general, secrete a range of biomolecules such as cytokines, chemokines, growth factors, interleukins, lipids, steroids, nucleic acids, ions, and metabolites, which can trigger physiological responses. The composition of the secretome released by each stem cell type, which besides different types of soluble factors also includes extracellular vesicles (EVs), is unique and cell-specific. Moreover, depends on the cell source, its differentiation status, and the environmental stimuli that induce a cell response to maintain cellular homeostasis. Cell donor-specific factors, such as age and health status, also affect the composition of the stem cell secretome since that significantly influence the quality of the donor stem cells. Due to the impact of all these factors, it is difficult to define a standard *in vitro* profile for the stem cells' secretome, which mostly relies on the conditioned medium (CM) obtained from the cultured cells (Zhang et al. 2016; Haider and Aramini 2020).

Indeed, studies already demonstrated that CM derived from MSC culture contributed to therapeutic effects, such as enhancement of wound healing (Yew et al. 2011), reduction of myocardial infarct size (Timmers et al. 2008), vascular remodeling and angiogenesis (Hung et al. 2007; Timmers et al. 2011), and improvement of acute lung injury (Ionescu et al. 2012). However, these multiple therapeutic effects are unlikely to result from a single molecular factor. Furthermore, since many of the proteins secreted do not have signal peptides, it is appropriate to consider their packaging along with other biomolecules, such as mRNAs and microRNAs (miRNAs), in membrane-bound vesicles.

EVs appeared as expected candidates to be released by MSCs as paracrine signaling, communicating therapeutic signals to the recipient cells and epigenetically regulating them to initiate repair and regeneration (Zhang et al. 2016; Matei et al. 2019). Compared with MSCs application itself, the application of EVs provides several advantages. In contrast to MSCs, EVs are non-self-replicative and due to their small size can be sterilized by filtration and easily reach the injured sites, traveling through blood without getting entrapped in the microvasculature when systematically delivered. Furthermore, EVs' surface structure and natural machinery allows an easy fusion with cells, which may induce a higher and longer therapeutic effect. Therefore, it is expected that EV-based therapeutic products are safer to administer when compared to the delivery of cellular therapies. (Kordelas et al. 2014; Xu et al. 2019).

1.2. Extracellular Vesicles

Direct cellular interaction and secretion of soluble factors mediate intercellular communication. More recently, there was increased evidence that EVs are a mean of cells to communicate with neighboring cells and with distant cells. EVs contain a large and complex cargo of DNA, RNA (mRNAs

and non-coding RNAs such as small regulatory miRNAs), proteins, lipids and metabolites, which can be released into the extracellular space and delivered to different cell types during both physiological and pathological conditions (Lee et al. 2012b; Kalra et al. 2016). Since these vesicles are released from most cells, they have been readily isolated from many biological fluids, including blood, urine, breast milk, saliva, amniotic fluid, bile, cerebrospinal fluid and semen. In general, EVs are composed of a lipid bilayer membrane and the enclosing cytosolic specific cargo of biomolecules. However, they can be classified according to their sub-cellular origin and depending on their biophysical properties (Colombo et al. 2014; Tkach and Théry 2016).

EVs can be divided into three subtypes according to their biogenesis, namely: exosomes; ectosomes or microvesicles; and apoptotic bodies. These subtypes have different biogenesis pathways, and both size and buoyant densities vary significantly, although it is noticed that the size and buoyant density ranges for the distinct subtypes have been heterogeneously reported in the literature. Some vesicles can also be identified by specific surface protein markers.

Exosomes are homogenous small particles around 30-150 nm in size, which have a buoyant density of 1.10–1.14 g/mL and exhibit cup-shape morphology when observed under transmission electron microscopy (TEM). These membranous vesicles are an end-product of the endocytic recycling pathway, being released into the extracellular space as a result of the fusion of multivesicular bodies with the plasma membrane (see Figure 1). Then, exosomes deliver their payload in the recipient cell by multiple mechanisms, for example, EV fusion with the cell membrane. The endosomal sorting complex required for transport (ESCRT complex) is involved in the exosome biogenesis, and enrichment of Alix, TSG101, CD9 and CD63 markers was already identified in this subtype of EVs. Furthermore, CD81 might distinctly be used as an exossomal marker (Lee et al. 2012b; Kalra et al. 2016). Microvesicles are included in a larger and more heterogeneous population of EVs that generally range from 100 to 1000 nm in diameter. Contrary to exosomes, they are released directly from the plasmatic membrane through outward budding off or protrusion (see Figure 1) and have no definite unique surface markers identified. However, microvesicles closely resemble the molecular composition of the cell membrane of origin (Lee et al. 2012b; Kalra et al. 2016). Apoptotic bodies are vesicles produced by cells undergoing apoptosis or programmed cell death and compose sizes around 50-5000 nm (Kalra et al. 2016).

Due to the composition of their cargo, EVs have a natural role in genetic information transfer between cells and have a significant impact on the physiological processes of the recipient cell (Lee et al. 2012b). Therefore, several studies have been focused on exosomes and microvesicles to exploit their potential for therapeutic applications and to provide a promising, safe and effective cell-free-based therapy that avoids the risk of uncontrolled cell division and tumor formation, possibly associated with the use of stem cells (Prockop 2010).

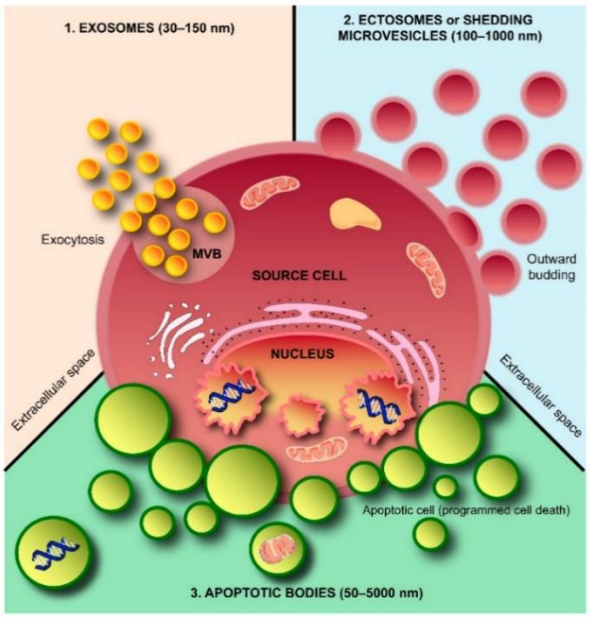

Figure 1.1- Schematic representation of the three subtypes of extracellular vesicles: exosomes, ectosomes or microvesicles and apoptotic bodies (Kalra et al. 2016). These subtypes differ in biogenesis pathways and size. Exosomes result from the fusion of multivesicular bodies with the plasma membrane and represent a small population of vesicles. Microvesicles are larger vesicles released directly from the plasmatic membrane through outward budding. Apoptotic bodies are produced in apoptotic cells and can reach the larger sizes of vesicles.

1.2.1. Therapeutic potential of MSC-derived EVs

MSCs have been suggested as the most promising cellular source of EVs to be therapeutically applied. According to the published studies about MSC-derived EVs, it seems that their action recapitulates in large part the MSC action reported in animal models of disease, including their homing to injured tissues ability (Kim et al. 2016). The therapeutic efficacy of MSC-derived EVs was firstly reported in 2009 by Bruno *et al.,* where MSC-derived microvesicles stimulated cell proliferation *in vitro* and conferred resistance of tubular epithelial cells to apoptosis, suggesting their protection against acute tubular injury (Bruno et al. 2009). Then, multiple groups reported that the smaller MSC-derived exosomes exhibit therapeutic potential for myocardial infarction (Lai et al. 2010; Arslan et al. 2013), renal protection (Zhou et al. 2013; Lindoso et al. 2014), also to protect the intestine from necrotizing enterocolitis (Rager et al. 2016) and to elicit hepatoprotective effects in damaged liver (Li et al. 2013; Tan et al. 2014). Exosomes derived from MSC were also able to reduce infarct size in mouse hearts and to reduce systemic inflammation in mice after myocardial ischemia/reperfusion (Ailawadi et al. 2015). Promising results using MSC-derived exosomes to treat refractory GvHD suggested that they may provide a potentially new and safe tool for the treatment of this disease and possibly other inflammatory associated diseases (Kordelas et al. 2014).

Furthermore, considering that exosomes can pass through the blood-brain barrier and can be engineered to contain a cargo of interest to target a desired tissue, make them good candidates to act as suppressors of pathological conditions in the central nervous system. Indeed, MSC-derived exosomes have produced beneficial effects in a variety of models of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease (reviewed in (Gorabi et al. 2019)). Stem cell-derived exosomes seem to produce neuroprotective effects through reducing oxidative stress, which is

important in neurodegenerative diseases considering that oxidative stress is a large contributor to disease progression. Exosomes obtained from human dental pulp stem cells were able to suppress apoptosis of dopaminergic neurons related to Parkinson's disease. Exosomes obtained from AT-derived MSCs decreased the pathological accumulation of β-amyloid in neuroblastoma cells associated with Alzheimer's disease. Furthermore, exosomes obtained from murine AT-derived MSCs showed enhanced neuroprotective effects in a *in vitro* model of amyotrophic lateral sclerosis. The use of exosomes secreted from BM-derived MSCs resulted in improved clinical outcomes in diverse neurologic disorders, including in the treatment of refractory GvHD, ischemic stroke and traumatic brain injury.

MSC-EVs were also therapeutic effective in diverse lung studies, exerting a pleiotropic protective effect and inhibiting pulmonary hypertension (Lee et al. 2012a), improving *Escherichia coli* endotoxin-induced acute lung injury (Zhu et al. 2014), restoring alveolar fluid clearance (Gennai et al. 2015) and attenuating radiation-induced lung injury (reviewed in (Xu et al. 2019)). EVs derived from MSC have also been studied as a potential therapeutic agent for the management of COVID-19 (reviewed in (Karn et al. 2021).

Besides the use for cancer diagnostic, many studies have revealed that exosomes could also be attractive for cancer therapy as new tools to transfer various molecules to cancer cells. Exosomes can deliver miRNAs that regulate physiological processes involved, for example, in the resistance of cancer cells to drugs. Exosomes derived from BM MSC and expressing miR-146 were shown to inhibit glioma cells growth. Exosomes can also be used to target tumors expressing the epidermal growth factor receptor, such as breast cancer cells, with nucleic acid drugs (Mirzaei et al. 2017).

1.2.2. Mode of action

MSC-derived EVs essentially act as transfer vehicles of proteins, lipids and nucleic acids between cells to trigger biological responses from recipient cells (see Figure 2). The elicited responses depend on the type of EV cargo, which influence the physiological behavior of the target cells by diverse mechanisms, including inducing therapeutic effects. For example, MSC-derived EVs were reported to contribute to the repair and recovery process of renal proximal tubular epithelial cells through the transfer of miRNAs involved in the renal recovery process. The transferred miRNAs downregulated coding-mRNAs associated with hypoxia, cytoskeleton reorganization, and apoptosis (Lindoso et al. 2014).

Considering the findings of the therapeutic potential of stem cells EVs in general, it seems that the therapeutic effects can occur through two different mechanisms. The injured tissues can release EVs to interact with and lead local stem cells to deliver therapeutic EVs to tissue repair. Or local stem cells close to the injured tissue release EVs that allow the tissue regeneration by leading the cells neighboring the injured tissues to de-differentiate and re-enter into the cell cycle (Lee et al. 2012b).Furthermore, considering the exosomes' ability to cross biological membranes and interact with a broad variety of cells, they can be re-engineered and their cargo modified to deliver a specific and desired payload to the target cells, such as miRNAs (Lee et al. 2012b; Haider and Aramini 2020).

It is also important to notice that as well-verified in the literature that therapeutic efficacy of MSC is inversely correlated with the developmental stage of the cell donor, the efficiency of EV production has been reported as inversely related to the developmental maturity of the cell donor. In many studies, MSCs derived from umbilical cord were least efficient in EV production compared with fetal tissue and

embryonic stem cell-derived MSCs, being the last ones the most efficient (Zhang et al. 2016; Haider and Aramini 2020). Moreover, some studies indicated that not all MSC-derived exosomes are equivalent. Exosomes released by MSCs derived from different sources can vary in the level of a certain factors and cargo composition, eliciting different physiological responses from recipient cells (Phinney et al. 2017).

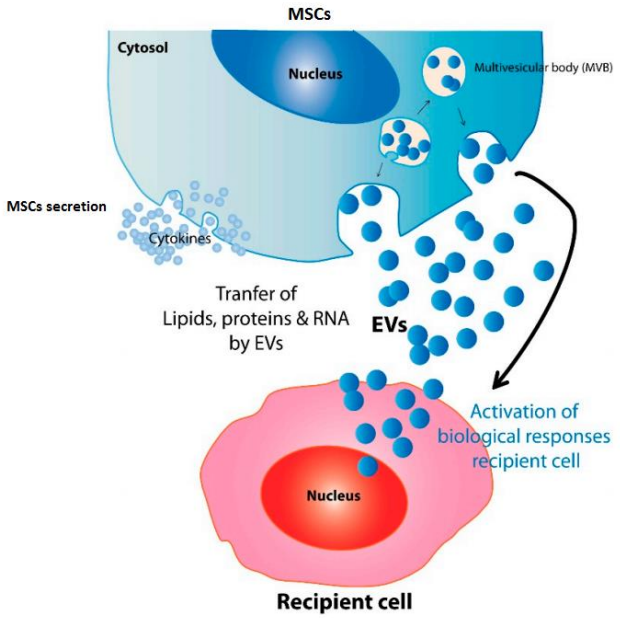

Figure 1.2- Schematic representation of the proposed mode of action underlying the therapeutic potential of extracellular vesicles secreted by stem cells (adapted from Zhang et al. 2016). EVs transfer biomolecules such as proteins, lipids and nucleic acids to recipient cells that trigger a physiological response through diverse mechanisms and possibly induce therapeutic effects.

1.3. MSCs as EV producers

The production of a given therapeutic agent requires the accomplishment of the Clinical Good Manufacturing Practice (cGMP)-grade standards for clinical use. These include consistency, uniformity, and reproducibility in the quantity and quality of the product. The high complexity of EVs, the high variability in their production and composition, as well as stability dependence on the vesicle source and preparation method, make the establishment of cGMP-grade to EV preparations especially challenging (György et al. 2015). Lately, several differences have been reported, in terms of the cargo of EVs (proteins and nucleic acids), between EVs isolated from culture supernatants of MSC expanded under different culture conditions, stressing the importance of controlling all culture process parameters to obtain consistent EVs content. Another reported aspect is the typical low yield of EVs. Therefore, in the production of MSC-derived EVs, it is important to choose the best cell source and understand the culture conditions under which the cells produce EVs with higher quality and quantity.

Regarding the cell source, MSCs can be obtained from several types of sources, including BM, AT and umbilical cord matrix (UCM), and can readily be expanded in culture. Human MSCs derived from BM have been the most extensively studied cell source and these cells are not tumorigenic. Therefore, it is unlikely the transfer of tumorigenic factors in the EVs they produce, a risk inherent with immortalized and embryonic cell lines.

AT and UCM, typically discarded as medical waste, have been more widely used as alternative MSC sources over the last decades. AT MSCs are isolated from lipoaspirates typically obtaining higher number of MSCs that grow fast when cultured under standard conditions (Kern et al. 2006; Peng et al. 2008). UCM MSCs are isolated from the umbilical cord units and are a more primitive cell source with lower immunogenicity and higher safety (Wang et al. 2016).

MSC immune-privilege and immunomodulatory potential allows the use of these cells as a ready-to-use and off-the-shelf product for an allogeneic setting, in which MSCs would be harvested from healthy and young donors, expanded and stored until further use (Richardson et al. 2013). Nevertheless, further studies should be performed to ensure the benefits of MSC therapy and limit immune rejection or other complications to the patients (Patel et al. 2008). Besides minimizing the risk of immune rejection that is the advantage of autologous therapy, allogeneic therapy also overcomes the disadvantages of autologous therapy, namely economical and time constrains of cell harvesting from patient own cells and expansion to achieve the necessary cell number. Furthermore, it overcomes the constrains of cell harvesting from elderly patients frequently with comorbidities whose cells may have lower regenerative potential and genetic abnormalities (Atoui et al. 2008; Zhuo et al. 2010; Nayan et al. 2011; Shin and Peterson 2012).

To produce enough quantity of EVs for clinical use (very large numbers of EVs are expected to be required; e.g., each patient may require $0.5 – 1.4 \times 10^{11}$ EVs (Kordelas et al. 2014)), it is necessary an adequate number of producing cells, typically of several million cells per kilogram of body weight (Elnakish et al. 2012). Since the MSCs isolated from the different cell sources have limited number of cells, *ex vivo* expansion of MSCs is required to reach a significant cell number for EV production. However, with extensive expansion and passaging MSC became senescent, plus it is an expensive and time consuming process with increasing risk of cell contamination and cell loss. (Zuk et al. 2001; Prockop and Keating 2012). These limitations can be overcome by selecting a more adequate cell source (for example, higher number of fast-growing cells is obtained from AT source), optimized culture conditions (for example, culture medium and oxygen tension) and culture systems (for example, bioreactor systems). As the culture conditions greatly affect MSC proliferation, multipotency and quality, it is important to perform the cell expansion at low density with a standardized protocol under which the cells retain most of their progenitor features. It is also important to preselect cells that expressed high levels of the biomarkers (for example TSG-6 mRNA) that were highly correlated with the efficacy of the MSCs in suppressing inflammation in study models and that provide information about the efficiency of MSC-EV production, such as CD63 (Sekiya et al. 2002; Kim et al. 2016).

Furthermore, the culture medium chosen should assure the best cell growth conditions. Most of the studies focused on scale-up culture systems used culture media supplemented with animal serum or serum replacement, which contains undefined xenogeneic factors (e.g. fetal bovine serum (FBS)) and extracellular matrix components from animal origin to promote cell attachment. However, they raise issues for therapeutic applications of MSCs, since transplantation of human cells exposed to animal-derived products presents a considerable risk of contamination of bacteria, animal proteins, virus, prions, immunogenic sugars, or xenogeneic antibodies that might trigger an immune response and chronic inflammation reactions. Likewise, their poorly defined composition results in inconsistent lot-to-lot

performance, introducing undesirable variability. Therefore, it is important to eliminate animal-derived components by using a fully defined serum-/xenogeneic free culture medium for the isolation and *ex vivo* expansion of human MSCs when targeting clinical applications (Santos et al. 2011; Simões et al. 2013; Akutsu et al. 2015).

1.4. MSCs isolation and expansion

MSCs isolation procedures are mostly based on the adherence to the plastic surface in culture dishes of the enrichment of mononuclear cell (MNC) fraction (Lee et al. 2005). Besides the cell source, the isolation method used also has an impact on cell quality and quantity. Possible protocols for MNC isolation from BM include density gradient centrifugation and gravity sedimentation (Lee et al. 2005; Romanov et al. 2005; Carrancio et al. 2008). It was already reported that gravity sedimentation can be more efficient in the isolation of MNC from BM compared with density gradient centrifugation. However, it can result in the loss of platelets that remain at the interface with the MNC in the density centrifugation isolation. Those platelets may secrete growth factors which are crucial to initiate cell proliferation, thus requiring human platelet lysate (hPL) supply to initiate the *in vitro* proliferation of MSC (Carrancio et al. 2008). After MSC isolation, an efficient *ex vivo* expansion is needed in order to have enough cells for EVs production, given the low frequency of MSC *in vivo*. Safety and potency of the expanded cells should be maintained to comply with Good Manufacturing Practices (GMP) guidelines and the processes used should be efficient and cost-effective, generating cells with well-defined characteristics in quantities that meet clinical demands.

1.4.1. Adherent static cell culture

MSC expansion at clinical-scale and in most academic laboratories has been traditionally performed under static two-dimensional adherent conditions using tissue culture flasks. To supply the growth factors necessary for cell expansion, these cultures are combined with supplements such as FBS and hPL. FBS has animal origin and is widely used for the *in vitro* cell culture of eukaryotic cells. hPL is a substitute supplement for FBS in experimental and clinical cell culture and result from human blood platelets after freeze/thaw cycles. The platelet lysis caused by the freeze/thaw cycle allows the release of a large quantity of growth factors including PDGFs, b-FGF, IGF-1, and TGFβ, which were shown to stimulate MSC proliferation. However, a decrease in osteogenic or adipogenic differentiation potential, with reduced immunosuppressive abilities, was already demonstrated in MSCs cultured with hPL. Furthermore, hPL and FBS are not fully defined media components since they are human- and animal-derived products, respectively, and their composition may vary between batches. Therefore, efforts were made to develop a well-defined culture medium free of human and animal-derived products. StemPro® MSC SFM is a xeno/serum-free product for MSC expansion which better complies the GMP guidelines and with reported ability to support cell proliferation and to maintain the cell's differentiation ability (Bunpetch et al. 2019).

Although 2D static planar technologies can combine scale-up and scale-out approaches, such as multiple-tray stacks and multiple units, these are still limited in terms of cell productivity and culture monitoring. This type of culture requires extensive handling and relatively long cultivation times,

increasing the risk of contamination and of undesired genetic abnormalities caused by multiple cell passages. Dynamic systems, such as perfusion and stirred tank bioreactors, have been developed as an alternative to expand MSC (Santos et al. 2011; Dos Santos et al. 2014).

1.4.2. Dynamic Cultures

MSC expansion processes, as well as culture systems in general, must offer standardized optimal growth conditions to produce cells with uniform properties and/or promote full monitored and controlled culture conditions while featuring easy scalability. Dynamic systems, including spinner flasks, stirred suspension vessels, rotating wall vessel bioreactors and perfused bioreactors, offer significant advantages over static culture standard flasks for *in vitro* MSC culture. They present ready scalability, a more homogeneous environment and the ability to monitor and control crucial culture parameters (such as temperature, agitation, pH, dissolved oxygen and metabolites) while reducing culture handling and costs to generate large numbers of functional MSCs that are needed to exploit their remarkable potential in therapeutic applications, including EVs production (King and Miller 2007; Eibes et al. 2010; Carmelo et al. 2015). These systems are usually combined with microcarrier culture, which provides large surface area for cell adhesion and growth, and importantly, a large surface/volume ratio (Badenes et al. 2016), being one of the most effective techniques to immobilize and cultivate adherent cells under stirred conditions. Macroporous gelatin microcarriers (Cultispher S) (Eibes et al. 2010), Plastic microcarriers, Synthemax® II and Enhanced Attachment® microcarriers (Carmelo et al. 2015) are examples of microcarriers that successfully supported MSC expansion in dynamic culture systems.

The fact that dynamic systems present the ability to control crucial culture parameters, facilitate their optimization to assure consistency and safety of the MSC-EV product. Different cell types and purposes will differ in the optimal parameter values (e.g. expansion versus differentiation). Considering that the production of MSC-EVs comprises MSC expansion, it is expected that the parameters that affect MSC expansion consequently affect EVs production, but that is still under investigation.

The agitation rate can be controlled in stirred systems to guarantee that an equilibrated homogeneous suspension of microcarriers is submitted to acceptable shear stress, avoiding cell damage and priming/inducing MSC differentiation into shear-responsive mesenchymal lineages, namely osteogenesis or chondrogenesis (King and Miller 2007; Dos Santos et al. 2014). Regarding oxygen tension, it is known that MSC growth and metabolism are affected by the concentration of oxygen available and therefore, the adequate oxygen transfer rate should be controlled by the aeration mode in bioreactors (sparging with gas bubbles or headspace delivery) and agitation, in order to favor MSC proliferation (Dos Santos et al. 2014). It has been demonstrated that human MSC grew more rapidly under a hypoxic environment (low O_2 concentration), which is closer to the physiological conditions, and exhibited greater retention of MSC markers and greater formation of colony-forming units (King and Miller 2007; Carrancio et al. 2008; Dos Santos et al. 2010). The adequate oxygen supply required for cell proliferation must be balanced against the detrimental effects of hydrodynamic shear stress developed in stirred and gas-sparged reactors. Furthermore, the adopted feeding scheme must assure the concentration of nutrients, metabolites and growth factors at non-inhibitory levels to maximize cell yield. The sensitivity to the build-up of metabolic byproducts and associated changes in pH must also be considered (King and Miller 2007; Dos Santos et al. 2014).

1.5. MSC-EV production

Different culture media have been used to culture MSCs, which include culture medium containing animal serum, culture medium supplemented with hPL and serum-free culture medium. The type of culture medium has impact on the cell culture and the regenerative properties of the cells. Moreover, that impact can also be reflected on the secreted EVs (Bobis-Wozowicz et al. 2017). Since EVs are isolated from MSC CM, the type of culture medium is an important parameter as it could be a source of contaminant EVs. Serum constitutes a source of contaminants EVs as it contains EVs with overlapping size to the EV population of interest. Thus, FBS-derived EVs will be co-isolated and any subsequent analysis will be biased (Szatanek et al. 2015). To circumvent this problem, filtration and long ultracentrifugation processes have been used to remove EVs from serum-containing media (Szatanek et al., 2015).

It has been demonstrated that FBS-derived EVs were able to promote migration of a lung carcinoma epithelial cell line (A549) and that contained RNA molecules. It was also recommended at least 18 h of ultracentrifugation at 100,000 relative centrifugal force to remove approximately 95% of RNA-containing FBS-derived EVs (Shelke et al. 2014). There is already EV-free FBS for purchase, however, it increases the costs of the cell culture process (Szatanek et al., 2015). It was also suggested the use of culture medium supplemented with 1% Bovine Serum Albumin (BSA) for the collection of CM for EV isolation (Théry et al. 2006).

Alternatively, serum-free culture media such as StemPro® MSC SFM medium can be used, which besides avoiding EV contamination, also avoids batch to batch variability and xenogeneic contaminants. EVs isolated from umbilical cord MSCs cultured in StemPro® MSC SFM medium were reported to enhance to a higher extent the proliferation and cardiac differentiation of human cardiac cells, to promote to a higher extent the formation of tube structures by endothelial cells, and inhibit mitogen-stimulated peripheral blood mononuclear cell proliferation when compared to other serum-free culture media and FBS-supplemented medium (Bobis-Wozowicz et al. 2017).

Even serum-free culture medium and freshly open medium bottles contain trace particles on the EV size range and the number of particles increases with storage time and temperature, which lead to an increase in the background when analyzing EV samples by nanoparticle tracking analysis or nano-flow cytometry (Szatanek et al., 2015).

Besides the culture medium, MSC-EV are affected by factors related to the culture techniques used, namely, cell passaging, culture conditions (temperature, oxygen tension, shear stress, stiffness, preconditioning with proteins or small molecules), methods of isolation (ultracentrifugation, chromatography, precipitation) and culture system (adherent in 2D or 3D, or spheroids). Additionally, MSC-EV characteristics are also dependent of the MSC donor characteristics, including donor age and sex, cell source, healthy donor or diseased patient and the presence of trauma or systemic diseases (Pountos et al. 2007).

1.6. Collection of EVs derived from MSCs

1.6.1. Isolation/Purification of EVs

Distinct EV subtypes have different mechanisms of biogenesis, different organelle origin, and different cargo. Therefore, to define the functionality and diagnostic/therapeutic applications of an EV subpopulation, it is important to guarantee their total isolation for a complete analysis of constituent molecules. Furthermore, considering the functional differences between EV subtypes, it is unclear how the use of impure samples in a clinical setting can overcome possible side effects (Xu et al. 2016). Currently, it is technically challenging to obtain a totally pure EV fraction free from non-vesicular components for functional studies, but efforts are being made to develop new methods or by applying a combination of several methods. The available methods include ultrafiltration, differential ultracentrifugation, density gradient ultracentrifugation, sucrose cushion centrifugation, gel-permeation chromatography, immunoaffinity chromatography, synthetic polymeric precipitation, microfluidics techniques and membrane filtration (Witwer et al. 2013; Xu et al. 2016; Nawaz et al. 2017). Although there is this long list of possible methods to achieve EV isolation, standardization is imperative to enable cross-laboratory comparisons and reproducibility of results. The sample type (for example, cell culture CM, milk, blood plasma, urine), the required degree of EV purity and the EV concentration are factors that should guide the choice of isolation method(s) used. In the case of EVs produced by MSCs, EVs are isolated from the CM after cell culture.

The International Society for Extracellular Vesicles (ISEV) has made an extensive analysis of the possible techniques for EV isolation and provided recommendations (Witwer et al. 2013). In 2018, ISEV established the minimal information for studies of extracellular vesicles providing information about EV collection, isolation and characterization and its reporting (Théry et al. 2018). Differential ultracentrifugation has been the most employed primary isolation method in the published studies of EV isolation from biofluids and cell culture, using or not size filtration to concentrate and partially purify EV populations. This technique is used for EV isolation of larger volumes of samples, however it has limitations when considering further scale up for the large scale EV production. Additional limitations of this technique include purity and particle aggregation (Witwer et al. 2013; Nawaz et al. 2017). Thus, this method is classified as having intermediate recovery and specificity (Théry et al. 2018). Diverse chromatographic techniques can also be used to separate EV based on their properties. Size exclusion chromatography is widely used for the isolation of EVs from low volume samples and allows a sequential elution of EV size fractions from a single column, separating EV from the bulk of soluble proteins. However, contaminating particles with the same size of the EVs of interest may be co-isolated (Nawaz et al. 2017). Immunoaffinity isolation offers an alternative method with a much higher selective specificity in which antibodies against surface proteins are used to capture the desired EV populations positively (immuno-enrichment) or to trap unwanted EV populations (negative selection or immuno-depletion). The EV yield is typically lower because markers are possibly not represented or recognized on all vesicles within a given class (Witwer et al. 2013). Therefore, this method is classified as having low recovery and high specificity. Microfluidic devices containing antibody-coated surfaces have been reported to rapidly recover small EVs from both serum and conditioned culture medium. There are also commercially

available kits that include polymeric precipitation mixtures, such as polyethylene glycol (PEG), which enables simple and rapid EV isolation from the CM or from body fluids without the need for specific and expensive equipments (as for the ultracentrifugation and chromatography methods). However, polymer-based precipitation methods result in low purity as it co-precipitates numerous non-EV contaminants such as RNA-protein complexes and lipoproteins (Witwer et al. 2013; Nawaz et al. 2017).

Considering that currently no method alone ensures high purity of the isolated EVs, a combination of at least two different isolation EV techniques based on distinct principles of separation is recommended by ISEV. For example, density gradient centrifugation can be followed by size exclusion or immunoaffinity capture (Witwer et al. 2013). Thus, each method enriches for different subpopulations of vesicles and separates the co-isolated contaminants. Besides the purity of EVs required for specific downstream analysis (proteomic or genomic), the choice of the method(s) should consider the sample type (derived from cell-culture media or from body-fluids), yield, volume, integrity, and the available instrumentation and processing time (Nawaz et al. 2017). It is also important to avoid cell disruption by the gentle collection of the EV-containing fluid to reduce the contamination with intracellular compartments, which would result in lower purity of the EV isolation.

1.6.2. EV characterization

To describe the molecular content or the functional consequences of the isolated material, it is necessary to determine the distinct contribution of EVs in any experiment, since the culture media and several body fluids contain non-vesicular entities such as extracellular RNA, lipoproteins and protein complexes that are co-isolated with EVs using the common isolation methods (including centrifugation or commercial kits) (Lötvall et al. 2014). There is a range of techniques available to characterize EV size, the presence of EV markers, EV morphology, and concentration after the isolation procedures (Nawaz et al. 2017). As referred before, ISEV proposed the minimal experimental requirements, based on current best-practice, for the definition/characterization of EVs and their functions (Théry et al. 2018). These guidelines promote transparency and standardization, while facilitating the experiment planning, results reporting and comparison between published studies. Subsets of EVs cannot yet be fully separated and distinguished by a list of “EV-specific markers” or physicochemical properties, but it is possible to differentiate between EV and non-EV components. Future improvements in the field could eventually allow an update in these criteria to provide specific markers and characteristics of EV subtypes.

ISEV minimal criteria and guidelines provide a general list of the proteins that should be analyzed for their presence or absence in the EV populations. Those proteins are divided into different groups: transmembrane or lipid-bound extracellular proteins, cytosolic proteins, and intracellular and extracellular proteins; and can be classified as expected (transmembrane proteins and cytosolic proteins with membrane-binding capacity) and not necessarily expected components to be present on or in EVs (e.g. proteins of endosomal origin). The description and amount of several proteins (3 or more) in at least a semi-quantitative manner in any EV preparation should be reported since different types of EVs seem to present variations in the relative proportions of different proteins (Théry et al. 2018). Researchers can also use databases (such as EVpedia or Vesiclepedia (Kalra et al. 2012; Kim et al. 2015)) to compare their protein isolates with those described in other EVs.

1.6.3. Methods of EV characterization

Methods used to identify and characterize EVs include scanning electron microscope (SEM), atomic force microscopy (AFM), flow cytometry, transmission electron microscopy (TEM), western blotting, nanoparticle tracking analysis (NTA) and ELISA.

The phenotypic analysis of EVs can be performed by flow cytometry, as well as sorting of EVs based on their size distribution, whereas submicron phenotype of EVs can be evaluated by electron microscopy.

EV-markers such as the tetraspanins (CD63, CD81, and CD9) are usually detected using Western blotting (Nawaz et al. 2017), but other techniques as nanoflow cytometry and ELISA could be performed. When analyzing EV population secreted by cultured cells, the level of enrichment of the EV components should ideally be determined by comparison with the secreting cells of origin. It is important to use the most adequate positive and negative controls, when characterizing EV populations. To evaluate the heterogeneity of the EV preparation, the use of single vesicle characterization methods is recommended (Lötvall et al. 2014). TEM and AFM can provide images showing a wide view of the vesicle population isolated as well as close-up images of single vesicles. Besides the heterogeneity, TEM also allows to determine EV morphology and purity (giving by the absence or presence of non-EV particles). NTA can assess the mean size and overall size distribution of EVs and can determine particle concentration. Although it is possible to distinguish heterogeneity based on the size, this technique does not distinguish membrane vesicles from co-isolated non-membranous particles of similar size. Therefore, NTA results should be compared with results from TEM, AFM or other imaging techniques (Lötvall et al. 2014; Nawaz et al. 2017; Chiang and Chen 2019).

There are also no guidelines and acceptable criteria regarding the characterization of all EV contents, namely lipids, metabolites and RNA. EV content can be characterized, for instance, by chromatography, mass spectrometry, high-field nuclear magnetic resonance or Fourier-transform infrared spectroscopy (FTIR).

In the case of functional studies to evaluate the effects of isolated EVs on target cells and their relationship to physiologic and pathological mechanisms, a quantitative analysis of the dose-function relationship should be presented, with additional information about the starting fluid volume or the number of producing cells used to isolate the range of functional EVs. The use of systematic negative controls is recommended by ISEV, to have a perception of the minimal functional effects. Other possible controls are EVs from unstimulated cells (when comparing with EVs isolated from cells under a stimuli), the full CM, the fresh culture media, or the fluid remaining after the EV isolation. Biochemical detection methods, such as qPCR, can be used to prove the association of certain molecules to EVs after fractionation of the EV preparation using density gradients. Antibody-mediated capture or depletion of EVs or the use of fluorescent labels of EVs incubated with target cells can be used as alternative approaches to link functional activity, or specific molecules, with isolated EVs from the biofluid or CM (Lötvall et al. 2014). However, it is still challenging to define the active components of EVs and their effects on target cells since, for example, it is difficult to identify the miRNAs with significant effects on their target cells between all the millions of potential targets obtained by sequencing of the miRNAs and other RNAs in EVs (Kim et al. 2016).

2. Contextualization and Aims of the study

EVs have been considered promising candidates to be used in diverse therapeutic settings. As intercellular communication mediators, transferring their cargo of proteins and RNA (i.e., microRNA and mRNA), these vesicles can trigger alterations on host cells. Due to their small size and identical structure to the cell membrane, EVs show ability to cross biological barriers and high biocompatibility to target cells. Furthermore, EV therapies bring the opportunity to develop cell-free therapies avoiding the complications associated with using cells themselves, since they can mimic some of the therapeutic effects from their cells of origin.

EVs derived from MSC are particularly interesting. Firstly, they are not expected to trigger immune reaction from the host immune system, as confirmed for MSC administration in several clinical trials, allowing the development of an allogeneic therapeutic product. And then, MSC-EVs present therapeutic properties that may be beneficial for a vast number of diseases, including cardiovascular, neurological, immunological, liver and kidney diseases. MSCs can be expanded *ex vivo*, using already established expansion platforms, however, further developments are needed to reach a large number of cells that secrete EVs in enough amount for clinical use. Furthermore, several differences have been reported in terms of the cargo of EVs (proteins and nucleic acids), between EVs isolated from culture supernatants of MSC expanded under different culture conditions, stressing the importance of controlling all culture process parameters to obtain consistent EV content. In this manner, culture parameters need optimization to ensure high productivity, combined with quality, consistency, reproducibility, and safety.

The main objective of this thesis was to study different cell culture parameters to obtain an optimized product of human MSC-EVs based on productivity. MSC-EV production comprised a step of MSC expansion followed by a step of cell conditioning after which the CM was collected and used for MSC-EV isolation. Three different experiments were designed in order to evaluate the effect of different parameters on MSC-EV production, namely, cell source, donor variability, time of EV collection, medium exchange (ME), culture medium composition, oxygen tension and temperature.

Alongside, it was investigated the presence of particles in the culture medium used and their possible impact in the quantification of MSC-EVs produced. To evaluate the influence of those particles, filtered and not filtered culture medium were compared. The possible EVs degradation and adhesion to plastic throughout cell culture was also assessed, as well as the MSC-EV uptake/production profile.

3. Materials and Methods

3.1. Procedure summary

MSCs used for the study of EV production were firstly isolated from human sources and expanded under static conditions. In the experiment entitled “time point experiment”, a total of nine different cell donors from three cell sources (BM, AT and UCM) were used to study EV production along culture time. Furthermore, the effect of different percentages of ME on EV productivity was also analyzed. The MSC-EVs produced were isolated from MSC CM and characterized in terms of protein concentration (by MicroBCA), particle number (by NTA), EV composition (by Western blotting and by FTIR) and in terms of morphology and purity (by TEM, and by the ratio between protein concentration and particle number). Additionally, after cell culture, the BM, AT and UCM MSCs were analyzed in terms of immunophenotype and multilineage differentiation capacity.

For the following experiments, BM was selected as a cell source to use.

The results from the time point experiment raise the question whether the vesicles present in the culture medium could affect the MSC-EV quantification. Therefore, in the second experiment, MSC-EV productivity was evaluated for cells cultured with filtered *vs* not filtered culture medium. In this experiment, only NTA analysis and protein quantification were performed.

Finally, in a third experiment, the effect of oxygen tension (normoxia *versus* hypoxia) and the effect of temperature (37ºC *vs* 33ºC) on MSC-EV productivity was assessed. NTA analysis and protein quantification were again performed for the EV samples isolated from MSC CM.

3.2. Human samples

All the human primary samples were obtained from healthy donors or patients after written informed consent according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of the respective clinical institution, according to the Portuguese Regulation (Law 21/2014, April 16). Human tissue samples were obtained from local hospitals and clinics under established collaboration agreements with iBB-IST (BM from Instituto Português de Oncologia Francisco Gentil, Lisboa; UCM from Hospital São Francisco Xavier, Lisboa, Centro Hospitalar Lisboa Ocidental, Lisboa; AT from Clínica de Todos-os-Santos, Lisboa). Human MSC used in this study are part of the cell bank available at iBB-IST and were previously isolated by plastic adherence according to established protocols (Gimble and Guilak 2003; de Soure et al. 2017; de Sousa Pinto et al. 2019).

3.3. Cell Culture

3.3.1. Mesenchymal Stromal Cells thaw and expansion in static conditions

The cryopreserved MSC from the different cell sources and cell donors were firstly removed from the liquid nitrogen cell bank at the Stem Cell Engineering Research Group (SCERG), iBB, and thawed in a water bath at 37°C for a short period (1-2 minutes). The content of each vial was carefully transferred into a falcon tube containing the appropriate amount of pre-warmed culture medium used for the cell freezing. Then, the cell suspension was centrifuged at 349 RCF (ScanSpeed 1580 MGR Centrifuge) for 7 min, the supernatant discarded and cells in the pellet resuspended in the appropriate amount of pre-warmed culture medium. The Trypan Blue (Gibco, Life Technologies) exclusion method was used to assess the cell number (**Equation 1**) and viability (**Equation 2**), using the dye trypan blue 0.1% and a haemocytometer under an optical microscope.

Equation 1- Cell number

$$Cell\ number = \frac{Number\ of\ cells}{Number\ of\ squares\ considered\ \ to\ the\ counting} \times dilution \times volume \times 10^4$$

Equation 2- Cell viability

$$Cell\ viability\ (\%) = \frac{Number\ of\ living\ cells}{Number\ of\ total\ cells\ (living + dead)} \times 100$$

After counting, cells were plated on Falcon® T-flasks pre-coated with CELLstart™CTS™ (Thermo Fisher Scientific), diluted in phosphate buffered saline (PBS) -/- (Thermo Fisher Scientific) 1:100 for 1 h at 37ºC, at a cell density around 3,000 cells/cm^2. If the culture medium used for cell expansion before freezing was not StemPro® MSC SFM XenoFree (Thermo Fisher Scientific), cells were thawed and expanded for one passage with the same culture medium and afterwards the culture medium was exchanged to StemPro® MSC SFM XenoFree. StemPro MSC culture medium that was composed of StemPro® MSC SFM XenoFree serum-free medium kit supplemented with 1% (v/v) Glutamax (Thermo Fisher Scientific) and 1% (v/v) antibiotic-antimycotic 100X (Thermo Fisher Scientific). Cells were incubated at 37ºC, 5% CO_2 and >95% humidity and the culture medium was replaced every 3-4 days. When reaching 70-80% cell confluence, cells were detached from T-flasks with xeno-free cell detachment solution TrypLE™ (1X, Thermo Fisher Scientific) for 5 min at 37°C. Cells were collected and diluted with the same amount of culture medium volume, centrifuged, resuspended in fresh culture medium and the cell number and viability were determined. MSC were passaged at least once using StemPro® MSC SFM XenoFree before the final inoculation in T-flasks for EV production under static conditions.

3.3.2. Metabolite analysis

For metabolite analysis, the CM samples were thawed and glucose and lactate concentrations were measured using an automatic analyzer (YSI 7100MBS; Yellow Spring Instruments).

3.4. MSC-EV production under static conditions

3.4.1. Time point experiment

To investigate the optimal time point for MSC-EV collection and the impact of different percentages of ME, previously cultured MSC from the different cell sources and cell donors listed in **Table 1.1** were passaged to T-175 flasks at 3000 cells/cm^2. For each cell source, MSC from three independent donors (n = 3) were used.

Table 1.1- List of donors from the different cell sources used in the time point experiment.

Source	Donors
Bone Marrow (BM)	M78A15, M79A15 and M86A15
Adipose Tissue (AT)	L090602, L090724 and L140326
Umbilical cord matrix (UCM)	UCM33(3), UCM46 and UCM81

Cells were cultured in the same conditions described before for MSC expansion under static conditions for 8 days, 6 days for cell expansion plus 2 days for medium conditioning. At day 3, culture medium was fully renewed, and at day 6 three different percentages of ME (0%, 25% and 100%) were exchange. Samples of CM were retrieved at days 3, 6 and 8, filtered with 0.45 µm filter to remove cell debris, and transferred to eppendorfs for further analysis (NTA and protein quantification) and to a falcon tube for EV isolation. These samples were stored at -80ºC until further handling.

At the end of the experiment (day 8), after the collection of the MSC CM, cells were detached from the flasks and the cell number was determined as previously described. Cells were re-suspended in culture medium and distributed for 1) flow cytometry analysis, 2) plated for multilineage differentiation assays, 3) cryopreserved at -80ºC using Cryostor for FTIR analysis and 4) pelleted and stored at −80°C for Western Blotting analysis.

3.4.2. StemPro *vs* filtered StemPro medium experiment

To evaluate if the particles present in the StemPro® MSC SFM XenoFree medium could affect the quantification of MSC-EVs produced, a comparison between MSC expanded with StemPro medium (SP) and StemPro medium filtered with 0.1 µm filter (SPf) and MSC-EV productivity was performed. BM MSC M79A15 were cultured in the same conditions described before for MSC expansion under static conditions for 7 days, 5 days for cells expansion plus 2 days for medium conditioning. The medium was totally exchanged at day 3 and two percentages of ME were studied at day 5, 0% and 100%. That was considered the necessary time to achieve around 80% of cell confluence. At day 7, the end of the experiment, the CM from the five T-75 flasks was recovered and filtered with 0.45 µm filter to remove cell debris. A portion of each CM sample was transferred separately to eppendorfs for NTA analysis and protein quantification. The rest was transferred to a falcon tube for EV isolation. The samples were kept in the freezer at −80ºC until further use. MSCs were detached from the T-flasks and cell number was determined as previously described.

Table 1.2- Percentages of medium exchange applied at day 5 in the culture medium filtration experiment; ME-medium exchange. SP- StemPro® MSC SFM XenoFree, SPf-filtered StemPro® MSC SFM XenoFree.

Medium exchange
0% ME of SP
100% ME of SP
0% ME of SPf
100 % ME of SPf

3.4.3. Hypoxia and temperature experiment

The effect of the cell culture parameters oxygen tension (normoxia *vs* hypoxia) and temperature (37ºC *vs* 33ºC) on MSC-EV production was also studied. For that, previously cultured MSCs from BM M78A15 donor were passaged to T-175 flasks, at 3,000 cells/cm^2. Cells were again cultured during 7 days, 5 days for cell expansion and plus 2 days for medium conditioning, under the conditions described in **Table 1.3**. The medium was not exchanged throughout MSC culture.

Table 1.3- Percentages of medium exchange applied at day 5 and conditions used throughout MSC culture in the hypoxia and temperature experiment. ME- medium exchange; SP- StemPro® MSC SFM XenoFree, SPf-filtered StemPro® MSC SFM XenoFree.

Medium exchange	MSC culture conditions
0% ME of SP 0% ME of SPf	Normoxia and 37ºC
0% ME of SP	Normoxia (21% O_2) and change from 37ºC to 33ºC at day 5 Hypoxia (5% O_2) and 37ºC
0% ME of SPf	Normoxia (21% O_2) and change from 37ºC to 33ºC at day 5 Hypoxia (5% O_2) and 37ºC

Samples of CM were retrieved from T-flasks at day 1, 3 and 5. At day 7, all CM was retrieved. CM samples were filtered with 0.45 µm filter to remove cell debris and a portion of each sample transferred separately to eppendorfs for NTA analysis and protein quantification. The rest of the CM volume was transferred to a falcon for EV isolation. The samples were kept in the freezer at -80ºC until further use. At the end of the experiment, MSC were detached from the T-flasks and cell number was determined as previously described. As a control, a sample of fresh culture medium was retrieved from the medium bottle in the beginning of the experiment and considered as sample from day 0. This sample underwent the same treatment as all other samples recovered from T-flasks.

Additionally, to investigate the presence of particles in the SP culture medium and the effects of particle adhesion to the T-flask and particle degradation during incubation at 37ºC, one T-75 flask with only SP culture medium (without cells) was incubated at 37ºC, 5% CO_2 and >95% humidity for 7 days. Samples were recovered at day 1, 3 and 7 for NTA analysis, protein quantification and EV isolation.

3.5. Isolation of EVs From MSC Conditioned Medium

EVs were isolated from the cells CM using the Total Exosome Isolation Reagent (Thermo Fisher Scientific). The CM samples were firstly thawed at room temperature and the isolation reagent PEG was added in a proportion of 1:2, PEG:sample. The mixture was pipetted up and down multiple times until the viscous PEG could not be distinguished, and incubated overnight at 4°C. The reagent addition decreases the solubility of components such as vesicles by tying up water molecules, allowing their sedimentation by a short, low-speed centrifugation (Konoshenko et al. 2018). Therefore, the mixture was then centrifuged for 1 h at 10000 × *g* and 4°C. The maximum volume of supernatant was removed carefully (without disturbing the EV pellet), and the EV fraction was recovered by thoroughly washing the walls of the centrifuge tube with DNase/RNase-Free PBS 1x prepared by mixing UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) and PBS +/+ 10X pH 7.4 (Thermo Fisher Scientific). EV samples were re-suspended in a PBS volume corresponding to a concentration factor of 30x to 60x relatively to the processed CM volume. EV samples were frozen at −80°C in aliquots for NTA analysis (40 µL of sample + 1960 µL of DNase/RNase-Free PBS 1x), protein quantification (24 µL of sample + 216 µL DNase/RNase-Free PBS 1x), and the rest of EV samples were stored in 100 µL aliquots.

3.6. MSC-EV characterization

3.6.1. Protein quantification

The Pierce™ Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) was used to quantify the total protein in EV enriched samples from all experiments, since it has been optimized for use with the diluted protein samples (0.5-20 µg/mL). In an alkaline environment, protein reduces Cu^{+2} into Cu^{+1}, which in this colorimetric method is detected by the agent bicinchoninic acid (BCA). The chelation of two molecules of BCA and one cuprous ion (Cu^{+1}) form a purple-colored reaction product that exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations (Smith et al. 1985).

The microplate procedure was followed according to manufacturer instructions. Firstly, EV and CM samples were thawed on ice. Then, diluted BSA Standards were prepared from a BSA Standard sample (2000 µg/mL) through successive dilutions using DNase/RNase-Free PBS 1x. The Micro BCA Working Reagent (WR) was prepared by mixing 25 parts of Micro BCA Reagent MA and 24 parts Reagent MB with 1 part of Reagent ME (25:24:1, Reagent MA:MB:ME). The EV and CM samples were diluted 10x with DNase/RNase-Free PBS 1x and 100 µL of each standard or sample were pipetted into a microplate well (in duplicates). The same quantity (100 µL) of the WR was added to each well and the plate mixed on a plate shaker for 30 seconds. After incubation at 37°C for 1h for CM samples and 1h30min for EV samples, the plate was cooled to room temperature and the absorbance measured at 562nm on a plate reader (Tecan i-control™). The average 562nm absorbance of the blank standard replicates was subtracted from the 562nm absorbance reading of all other individual standards and sample replicates. A linear fit was applied to the BSA standards and sample concentration was determined using the resulting equation to determine each sample concentration from its absorbance measurement.

3.6.2. Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) allowed to obtain the EV size distribution profiles and concentration measurements, using a NanoSight LM14c instrument equipped with a 405 nm laser (Malvern) and NTA software version 3.1 (Malvern). NTA is more suitable for the polydisperse EV samples analysis due to the fact that it can simultaneously analyze a population of nanoparticles on an individual basis. This technique to determine the particle size distribution of samples in liquid suspension merge the properties of both light scattering and Brownian motion. The particles in suspension that pass through the sample chamber scatter light of an incident laser beam. This allows them to easily be visualized via a 20x magnification microscope onto which is mounted a camera. A video file of the particles moving under Brownian motion is captured by the camera which operates at approximately 30 frames per second (fps). The measurement of particles Brownian motion allows the determination of nanoparticle size by NTA. The concentration measurement relies on an average that results from data from all the recorded frames and that is divided by the volume of sample in which the number of particles is measured. To assure statistical robustness, the number of particles analyzed within the sample time must be adequate (Malvern Instruments Limited 2015).

In the time point experiment, NTA acquisition and processing settings varied slightly between samples, while for the other experiments settings were selected based on that and kept constants for all samples. EV and CM samples were analyzed with a final concentration in the range of 10^8 to 10^9 particles/mL after dilution with DNase/RNase-Free PBS 1x in a final volume of 2 ml. Samples from the time points experiment were measured using a camera level between 10 and 13. Samples from the other experiments were analyzed with a camera level of 13. Each sample was recorded 15 times for 15 s, using fresh sample for each acquisition (by injecting sample contained in the syringe, in the NTA sample inlet). PBS was used to thoroughly wash the detection chamber between each sample measurement. A threshold level of 7 was applied for video processing. Each video recording was analyzed to obtain the size and concentration of EVs.

3.6.3. Western Blotting

Previously frozen cells from the time point experiment were thawed on ice and then lysed with Catenin lysis buffer (1% Triton X-100, Sigma, 1% Nonidet P-40, Sigma, in PBS) supplemented with protease inhibitor (7x, Sigma) and phosphatase inhibitor (100x, Sigma). Around 1M cells in suspension were mixed with lysis solution in the ratio of 1:3 (lysis solution:cell suspension) and kept on ice for 10 min. Then, vortexed 3 x 10s and centrifuged at 14000 × *g* for 10 min at 4°C to remove insoluble material. Supernatants were recovered and used as whole cell lysates (WCL). MicroBCA kit was used to quantify total protein content in WCL and EV samples as described before (1h of incubation at 37ºC for WCL samples).

The solutions to be loaded in the gel were prepared on ice mixing both WCL and EV samples with LSD sample buffer and reducing agent. Then solutions were heated to 100°C for 10 min in Thermomixer® comfort (Eppendorf AG). Protein ladder Spectra Multicolor Broad Range (5 µL) and all samples were loaded (15–60 µg of total protein) in Bolt™ 4–12% Bis–Tris polyacrylamide gels (Invitrogen, Life Technologies), in equal protein content for each gel, and subjected to electrophoresis.

A transfer module Power Blotter System (Thermo Fisher Scientific) was used to transfer the proteins into nitrocellulose membranes. To confirm the protein transfer, Ponceau Dye (Thermo Fisher Scientific) was used to stain the proteins and observe them. After extensive washing to remove the Ponceau Dye, the membranes were cropped to separate the regions where different proteins were expected to be detected by different antibodies. Membranes were blocked with 5% BSA solution in tris-buffered saline (TBS) Tween 20 buffer 1x (Thermo Fisher Scientific) for 1h with mild orbital agitation at room temperature and incubated with primary antibodies overnight at 4°C. In the following morning, membranes were washed with TBS Tween 1X, 3x 5min, and then incubated with HRP conjugated secondary antibodies for 1h at room temperature with mild orbital agitation. Primary antibodies were diluted with BSA blocking solution while secondary antibodies were diluted with TBS Tween (listed on **Table 1.4**).

For revelation, Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) was applied to the membranes and image acquisition was performed on iBright™ CL1500 Imaging System (Thermo Fisher Scientific).

Table 1.4- List of primary and secondary antibodies used for Western Blotting and respective dilutions.

	Antibody	Dilution
Primary	Anti-CD63 (Genetex)	1:1000
	Anti-CD81 (Abcam, ab109201)	1:500
	Anti-TSG101 – (BD, 612696)	1:500
	Anti-Calnexin (BD, 610523)	1:1000
	Anti-Synthenin (Abcam, ab133267)	1:1000
	Anti-GAPDH (Santa Cruz)	1:1000
Secondary	Goat anti-Mouse IgG (Invitrogen, Life Technologies, G-21040)	1:5000
	Goat anti-Rabbit IgG HRP-conjugated (R&D Systems, HAF008)	1:1000

3.6.4. Fourier-transform infrared spectroscopy

The molecular composition of CM and cell samples from the time point experiment were analyzed by Fourier-transform infrared spectroscopy (FTIR). The CM samples were thawed and added in triplicates of 25 µL to a 96 FTIR well plate. Cells cryopreserved with Cryostor solution at -80ºC were thawed and diluted in SP medium. After centrifugation, supernatant was removed and cells resuspended in PBS for counting. Triplicates of about 70,000 total cells per well were added to the 384 FTIR well plate (in 5-8 µL per well). Furthermore, triplicates of SP medium and PBS solution were also added as controls, as well as internal FTIR controls. FTIR analysis was performed at Instituto Superior de Engenharia de Lisboa under a collaboration with professor Cecília Calado's group.

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3.6.5. Transmission electron microscopy

TEM was used to detect and characterize single vesicles within EV samples. EV-enriched populations previously isolated and stored at -80ºC, were thawed and imaged by TEM by a technician at Instituto Gulbenkian da Ciência. For TEM, it was used the Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI) operating at 120kv, and data was collected with an Olympus-SIS Veleta CCD Camera. Imaging was performed following negative staining protocol. In summary, a 100 Mesh copper grids were coated with formvar and carbon, and glow discharged right before use. EV samples were mixed (1:1) with formaldehyde 4% in 0.1 M phosphate buffer (final concentration 2% formaldehyde in 0.05 M phosphate buffer) and incubated for 5 min at room temperature. Then samples were added to the grids and were incubated for 5 min to promote EV adhesion to the grids. Next, washing in 10 drops of distilled water was performed and samples were stained in 2 drops of uranyl acetate 2% by incubation for 5 min at room temperature in the dark followed by sample imaging.

3.7. MSC characterization

3.7.1. Flow cytometry

Immunophenotypic analysis of the cells was performed after the time point experiment. Flow cytometry was used to evaluate the expression of the surface markers CD14 (PE anti-human IgG1, clone MOP9), CD19 (PE anti-human IgG1, clone HIB19), CD31 (PE anti-human IgG1, clone WM59), CD34 (FITC anti-human IgG1, clone 581), CD45 (PE anti-human IgG1, clone HI30), CD73 (PE anti-human IgG1, clone AD2), CD80 (PE anti-human IgG1, clone 2D10), CD90 (PE anti-human IgG1, clone 5E10), CD105 (PE anti-human IgG1, clone SN6) and HLA-DR (PE anti-human IgG2a, clone L243). IgG1 κ (PE, clone MOPC-21) and IgG2a κ (PE, clone MOPC-173) were used as isotype controls. All antibodies for flow cytometry were acquired from Biolegend with exception of IgG1 and CD14 that were purchased from BD Biosciences and CD105 that was acquired from Thermo Fisher Scientific. Per antibody reaction about 100 x 10^3 cells were used. Cells were centrifuged at 349 RCF for 7 min and the pellet was resuspended in PBS and incubated for 15 min at room temperature with antibodies diluted 1:20 in a total of 100 µL, light protected. After incubation, 2 mL of PBS were added and cells suspension was centrifuged at 224 RCF for 5 min. The supernatant was discarded, and the cells were resuspended in 500 µL of 1% paraformaldehyde (PFA, Sigma-Aldrich), a cell fixative solution. Cells were stored at 4ºC, light protected, for up to one week when not immediately analyzed.

A minimum of 10 000 events were measured for each sample, and the data was acquired using FACSCalibur with CellQuest software (Becton Dickinson) and analyzed by FlowJo software (FlowJo).

3.7.2. Multilineage differentiation assays

MSC multilineage differentiation capacity from BM, AT and UCM MSC used in the time point experiment were also assessed by immunostaining after induction with osteogenic, adipogenic or chondrogenic differentiation medium.

Adipogenic differentiation

BM, AT and UCM MSC detached from T-flasks were plated on CELLstart CTS-precoated 24-well plates using StemPro MSC SFM XenoFree. About 100,000 cells were plated per well. At 80% cell confluence, supernatant was removed and adipogenic differentiation was induced with StemPro Adipogenic Differentiation medium (Thermo Fisher Scientific) for 14 days. The medium was changed three times a week (300-350 µL per well). After that time, staining was performed using Oil Red-O solution (Sigma). Supernatant was removed, cells were washed with PBS and incubated with 300 µL PFA 4% 20 min at room temperature for fixation. After fixation, cells were washed again with PBS and incubated with isopropanol solution 60% (Sigma) for 5min at room temperature. Then, cells were incubated with Oil Red-O solution (Sigma) (0.3% in isopropanol) at room temperature for 1h (300µL/well). After, cells were washed four times with distilled water and left in PBS. The assessment of differentiation toward an adipocytic phenotype was performed based on the accumulation of lipid vesicles, using the microscope Leica DMI3000 B.

Osteogenic differentiation

BM, AT and UCM MSC detached from T-flasks were plated on CELLstart CTS-precoated 24-well plates using StemPro MSC SFM XenoFree. About 100,000 cells were plated per well. At 80% cell confluence, supernatant was removed and osteogenesis was induced using StemPro Osteogenesis Differentation medium (Thermo Fisher Scientific). The medium was changed around three times a week for 14 days. After differentiation induction, supernatant was removed, the cells washed with PBS and fixed in 300 µL PFA 4% at room temperature for 20 min. Afterward, cells were washed again with PBS and incubated for 40 min at room temperature with Alkaline phosphatase solution (ALP) (300µL/well) containing Naphtol AS MX-PO4 (0.1 $mg \cdot mL^{-1}$) (Sigma) in 0.6 $mg \cdot mL^{-1}$ of Fast Violet LB salt (Sigma). Then, cells were washed four times with distilled water and incubated with silver nitrate (2.5% w/v) (Sigma) (300µL/well) for von Kossa staining, 30min at room temperature. Finally, cells were washed again with distilled water and left in PBS. The microscope Leica DMI3000 B was used to observe cells and assess differentiation toward an ostogenic phenotype based on the visualization of osteogenic progenitor cells and the accumulation of calcium deposits.

Chondrogenic differentiation

For chondrogenic differentiation induction, detached BM, AT and UCM MSC were centrifuged, the supernatant was removed, and cells were resuspended in about 200 µL to make around 10 small droplets of 20 µL with high cell densities (~$2x10^6$ $cells \cdot mL^{-1}$) in the caps of Petri dishs (Corning). The lower part of the dishes was filled with 4 mL of PBS + 2% anti/anti, the caps were rapidly flipped with the droplets to close them and incubated overnight at 37ºC with cells as hanging droplets. On the next day, the cell aggregates of the handing droplets were collected and placed in ultra-low attachment 24

well plates filled with 400 µL of Mesencult™- ACF Chondrogenic Differentiation medium (StemCell™ Technologies) (around five aggregates per well). The medium was changed three times a week for 14 days. After that time, supernatant was removed, and cells washed with PBS to be fixed in 300 µL PFA 4% at room temperature for 20 min. Then, cells were washed with PBS, incubated with 1% Alcian Blue solution (Sigma-Aldrich) at room temperature for 1 h, washed again and imaged. The synthesis of proteoglycans by chondrocytes was assessed under the microscope Leica DMI3000 B to verify the differentiation toward a chondrocytic phenotype.

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.2.0 Software. Results are presented as mean ± standard error of the mean (SEM) of the values obtained from different MSC donors (i.e., biological replicates) or as mean ± standard deviation (SD) of the values from technical replicates.

4. Results and Discussion

For the development of cell-based therapies, one of the important aspects is the use of appropriate cell culture medium. Culture medium supplements for *ex vivo* expansion based on animal derived products (including human), such as FBS and hPL, although being very useful for easily growing MSCs, they present several disadvantages. There are concerns regarding suitability and safety, including the risk of transmission of diseases by viruses and the possibility of provoke immunological responses (Hemeda et al. 2014). It is crucial to remove serum and xenogenic components, at least during the EVs production and harvest phase, to safeguard safety and facilitate approval of the therapeutic products (Witwer et al. 2019). Furthermore, this type of media supplements is not fully defined, and thus may suffer variation in composition between batches.

StemPro® MSC SFM XenoFree culture medium, a well-defined xeno/serum-free product specially formulated for the growth and expansion of human MSCs, was used in this work for MSC expansion to avoid the disadvantages mentioned above. TrypLE, also an animal product-free, was used as a cell detachment solution. Regarding the cell culture platform, static culture systems (i.e., T-flasks) were chosen to study different culture parameters such as cell source, donor, time of EV collection, medium exchange, culture medium composition, oxygen tension and temperature.

4.1. Time point experiment

4.1.1. MSC Expansion and Medium Conditioning for MSC-EV Production

In a first experiment, MSC isolated from three different human tissue sources, BM, AT and UCM, were used to study the optimal time point for EV collection and the influence of different percentages of ME at day 6 of culture in MSC-EV production, 0%, 25 % and 100 % ME. For each cell source, MSCs from three independent donors (n = 3) were successfully expanded under static conditions in T-flasks using StemPro XenoFree culture medium, while maintaining high viability. At the beginning of the experiment, around 525 000 cells were plated per T-175 flask for each condition of ME and the cells were counted again when detached at the end of experiment (day 8) (**Figure 4.1**). Compared to BM and AT MSCs, UCM MSC showed relatively higher number of cells, ranging between (19 ± 0.60) × 10^6 and (34 ± 3.2) × 10^6 depending on ME percentage. BM and AT MSC reached similar cell numbers, ranging between (13 ± 2.9) × 10^6 and (20 ± 2.2) × 10^6, and between (13 ± 2.7) × 10^6 and (19 ± 1.2) × 10^6, respectively. The higher proliferative capacity of UCM MSCs could potentially be explained by a more immature state of this cell source and smaller cell size, when compared to the adult BM and AT sources. Furthermore, the ME at day 6 seemed to have influenced the final cell number, since as higher was the percentage of ME, the higher was the total cell number at day 8 for the three sources, but particularly higher for UCM MSCs. However, the differences in cell number between percentages of ME did not seem statistically significant. Even so, the ME may have allowed the cells to continue to grow healthy and avoid cell death, more considerably in the case of 100 % than 25 % ME. The lower cell number for the 0% ME, and even for the 25% ME condition, could be explained by higher cell death or lower cell replication due to the metabolite concentration in the culture medium. In fact, the glucose levels were

exhausted by day 8 for BM and UCM MSCs for 0 and 25% ME and for AT MSCs the glucose concentration was also quite low (**Supplementary Material 7.1**). Similarly, high levels of lactate were produced for most of the donors from the three cell sources for 0 and 25% ME, compared to 100% ME (**Supplementary Material 7.2**). Despite of the high levels of lactate, it did not reach the inhibitory concentration for MSC culture (35.4 mM or 3.19 g/L) (Schop et al. 2009).

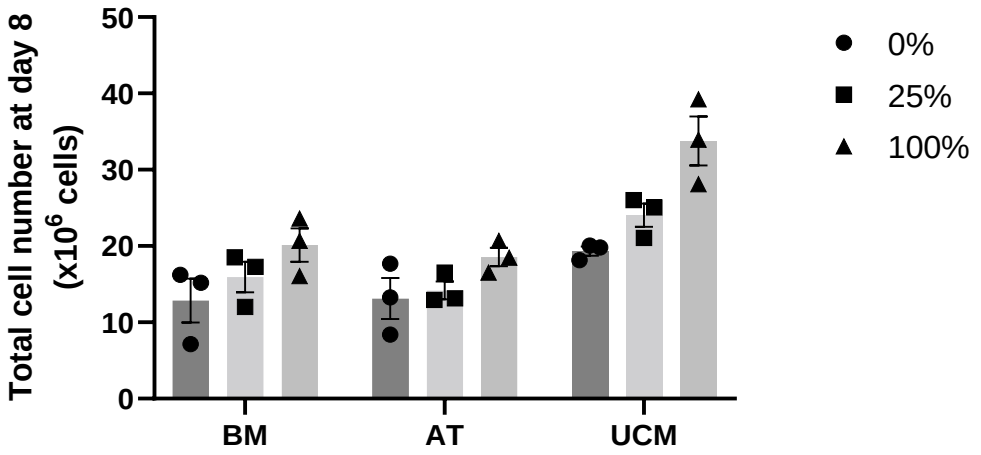

Figure 4.1- Total cell number (x10^6 cell) at day 8, the end of the time point experiment. Six days of MSC expansion plus 2 days of medium conditioning, for MSCs from three different human tissue sources (BM, AT and UCM) cultured under static conditions. MSC from three different donors were used per tissue source and three different percentages of medium exchange at day 6 were investigated, which are represented in three different shades of gray. To reveal the variation across the measurements, these data are plotted as individual points (0 % (circle), 25% (square) and 100 % (triangle) of medium exchange) and the average of these measurements is also shown. Results are presented as mean ± SEM of cell count for each percentage of medium exchange. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

After this experiment, immunophenotypic analysis of the cells was performed using flow cytometry to evaluate if the different percentages of ME affected the expression of MSC surface markers. According to the minimal criteria for defining multipotent MSC (Dominici et al. 2006), more than 95% of the cell population should express the specific surface marker CD73, CD90 and CD105, and less than 2% of cell population can be positive for the negative markers, CD11b, CD14, CD19, CD34, CD45, CD79α and HLA-DR. There was an unexpected increase in CD34 for AT MSCs and a decrease in CD105 expression of BM MSCs, which might be due to the antibody or to longer times of exposure to the cell detachment reagent (de Sousa Pinto et al. 2019) or due to the several passages (Dominici et al. 2006). Nevertheless, the percentage of ME did not greatly affect the expression of the markers, as represented in **Figure 4.2A** (and showed as values in **Supplementary Material 7.3**). Additionally, BM, AT and UCM MSC cultured in static conditions retained their multilineage differentiation ability towards the osteogenic, adipogenic and chondrogenic lineages, being able to differentiate into the 3 lineages although to a still immature state for the different percentages of ME studied, as represented for AT 326 for 25% ME in **Figure 4.2B, Figure 4.2C** and **Figure 4.2D,** respectively**.** Additionally, MSC have a spindle-shaped, triangular shaped and fibroblastic like morphology, according to what is expected for MSC (Liu et al. 2016). Altogether, human MSC from the different cell sources and cell donors were characterized by their plastic adherence, surface marker expression and differentiation potential according to the ISCT (Dominici et al., 2006).

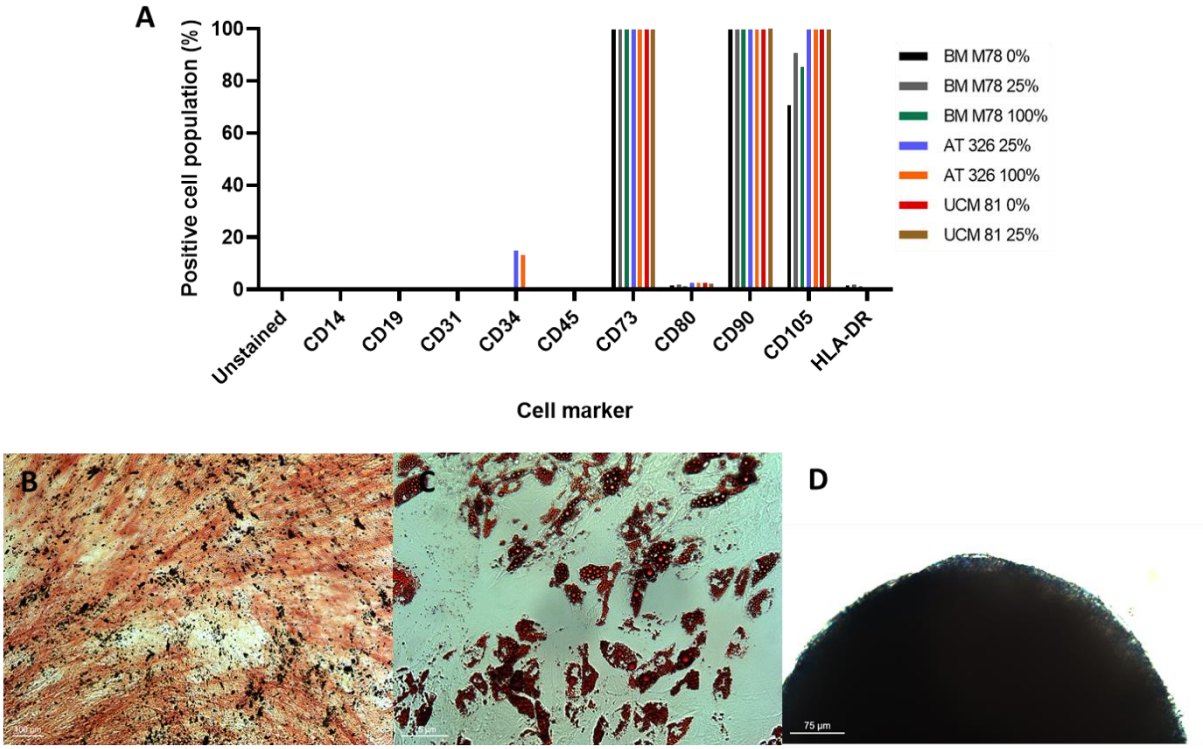

Figure 4.2- Analysis of MSCs obtained after the time point experiment. **(A)** Immunophenotypic characterization (% of positive cell population) of BM, AT and UCM MSC after expansion and medium conditioning for MSC-EV production (total of 8 days) in static culture, analyzed by flow cytometry. One donor was used for each cell tissue source, corresponding to all percentage of medium exchange studied (0%, 25% and 100%) for BM MSC, to 25% and 100% of medium exchange for AT MSC and to 0% and 25% of medium exchange for UCM MSC. Representative images of multipotency characterization of AT 326 MSCs - 25% ME, cultured in static system through multilineage differentiation assays for 14 days and assessed by staining for **(B)** osteogenesis (ALP and von Kossa), **(C)** adipogenesis (Oil Red-O), and **(D)** chondrogenesis (Alcian blue).

4.1.2. Characterization of MSC-EVs produced throughout experiment

Throughout culture time, conditioned medium (CM) samples were harvested at different time points (days 3, 6 and 8) and EVs were successfully isolated with a commercially available reagent based on PEG precipitation.

The presence of MSC EVs from the three different MSC sources was identified by TEM imaging (**Figure 4.3A, B** and **C**). Several individual EVs were identified showing different sizes around 100 nm, which is within the range of values obtained by NTA analysis (typically between 80-250 nm, shown further ahead in **Figure 4.7**). However, due to the sample processing techniques (fixation and dehydration), EV size and morphology (EVs presented a cup shaped with a divot in their center) were affected, as reported already in the literature (Doyle, L. M., & Wang 2019; Twain 2020). Furthermore, the samples seemed to contain other type of particles and filament-like structures beyond the EVs identified, indicating that EV samples were not pure (as confirmed also further ahead by purity assessment).

Western blotting analysis was also performed to confirm the presence of EVs (**Figure 4.3D**). Three different categories of proteins were tested according to the minimal experimental requirements for definition of extracellular vesicles (ISEV guidelines, (Théry et al. 2018)). As expected, the EV protein markers CD63 and synthenin (key proteins involved in EV biogenesis) were successfully detected in EV samples as well as in WCL, as transmembrane and cytosolic protein EV markers, respectively. Calnexin,

a negative EV protein marker for being an intracellular protein from the endoplasmic reticulum, was identified in cells and not detected in EV samples, as expected (Théry et al. 2018; de Almeida Fuzeta et al. 2020). Although for this analysis were used representative EVs samples obtained from BM, AT and UCM MSC cultured under static conditions for 8 days with completely medium exchange (100% ME) at day 6, similar results were obtained for 0% ME (data not shown). Thus, it is plausible to assume that also for the 25% ME using the same isolation method, it is possible to isolate MSC-EVs with the expected markers.

The TEM and Western Blotting results indicate that MSC-EVs were successfully produced in static culture system using StemPro culture medium and isolated using the PEG precipitation method.

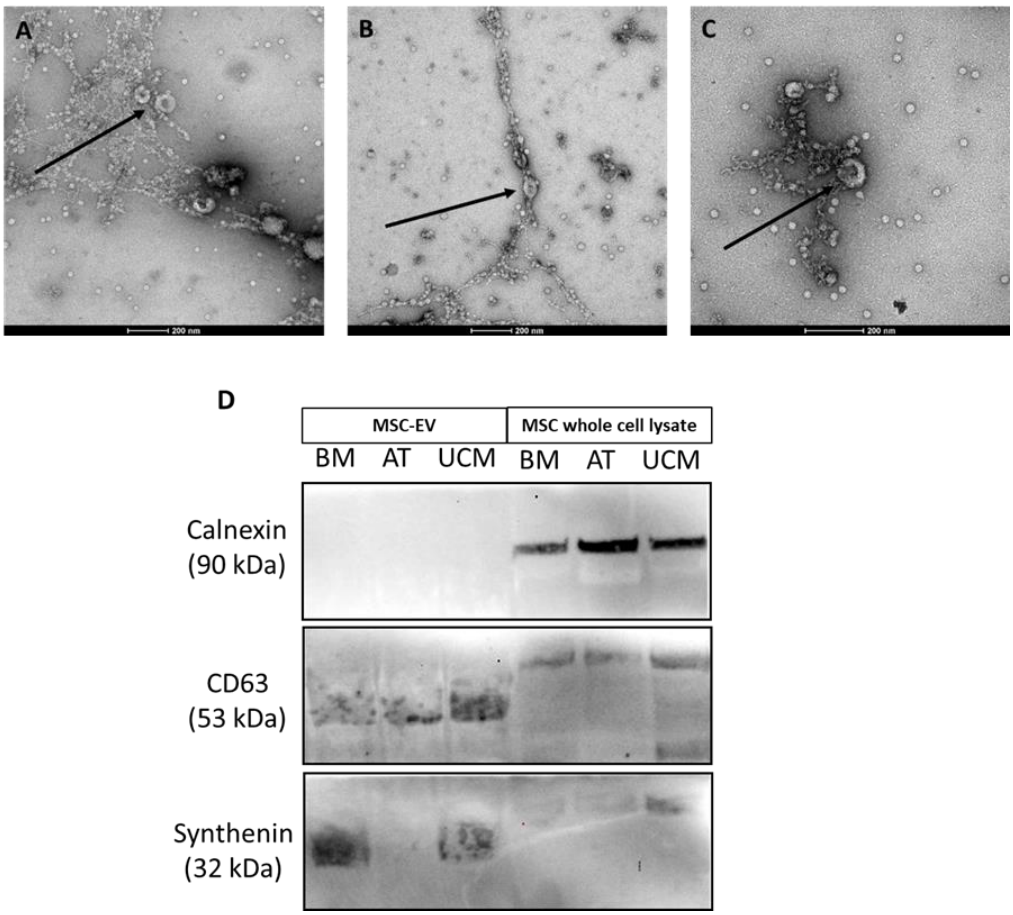

Figure 4.3- Characterization of MSC-EVs. Representative close-up TEM images of MSC-EVs obtained in static culture (samples from day 8 of time point experiment), using MSCs from three different human tissue sources (**(A)** bone marrow, **(B)** adipose tissue, and **(C)** umbilical cord matrix). EVs are pointed with black arrows. **(D)** Representative Western blotting detection of calnexin, CD63 and synthenin in MSC-EV samples and corresponding WCL (i.e., cells), obtained from BM, AT and UCM MSC after EV production in static culture. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

The molecular composition of CM and cell samples from the time point experiment was analyzed by FTIR. Furthermore, a PCA analysis was performed to compare the effect of diverse parameters, including the day of CM sample harvesting, cell source and percentage of medium exchange applied. Firstly, observing the molecular profile of fresh StemPro medium that was not in contact with cells, it is notorious that there are significant differences comparing to the CM (**Figure 4.4A**), indicating that the cells consume molecules from the culture medium and secrete other elements to it. It was also observed significant differences in the composition of CM obtained at different days of culture (day 3 *vs* day 6 *vs*

day 8), regardless of the cell source (**Figure 4.4A**). Curiously, the differences between the culture media obtained on different days (dispersion of points from different colors) is greater than the differences observed between media from different sources (dispersion of points from the same day, same color), indicating that there was an evolution in the molecular composition of CM throughout the experiment, possibly related to the cell growth and replication, cell metabolism and cell communication (EVs, growth factors, cytokines and other soluble factors produced by MSCs).

The PCA obtained for cell samples from the different tissue sources at day 8 of experiment (**Figure 4.4C**) showed a distinct molecular profile tendency from AT MSC (red circles) comparing with BM and UCM MSC samples (blue squares and green triangles, respectively), which was according to the observed in CM samples (**Figure 4.4B**). Apparently, it was also identified some differences between the molecular composition of BM and UCM CM samples (**Figure 4.4B**- blue squares and green triangles, respectively), but these differences were not evident in the PCA of cell spectra (**Figure 4.4C**). The differences in the molecular profile of CM and cells from the three cell sources were expected since MSCs have inherent differences associated to the tissue of origin and are conditioned by environment in which they are originated from. In this manner, it is also expected that EVs obtained from cells derived from each MSC source will have different molecular profile and functional characteristics (Ribeiro et al. 2013; de Almeida Fuzeta et al. 2020), reflecting the differences observed in the molecular composition of CM.

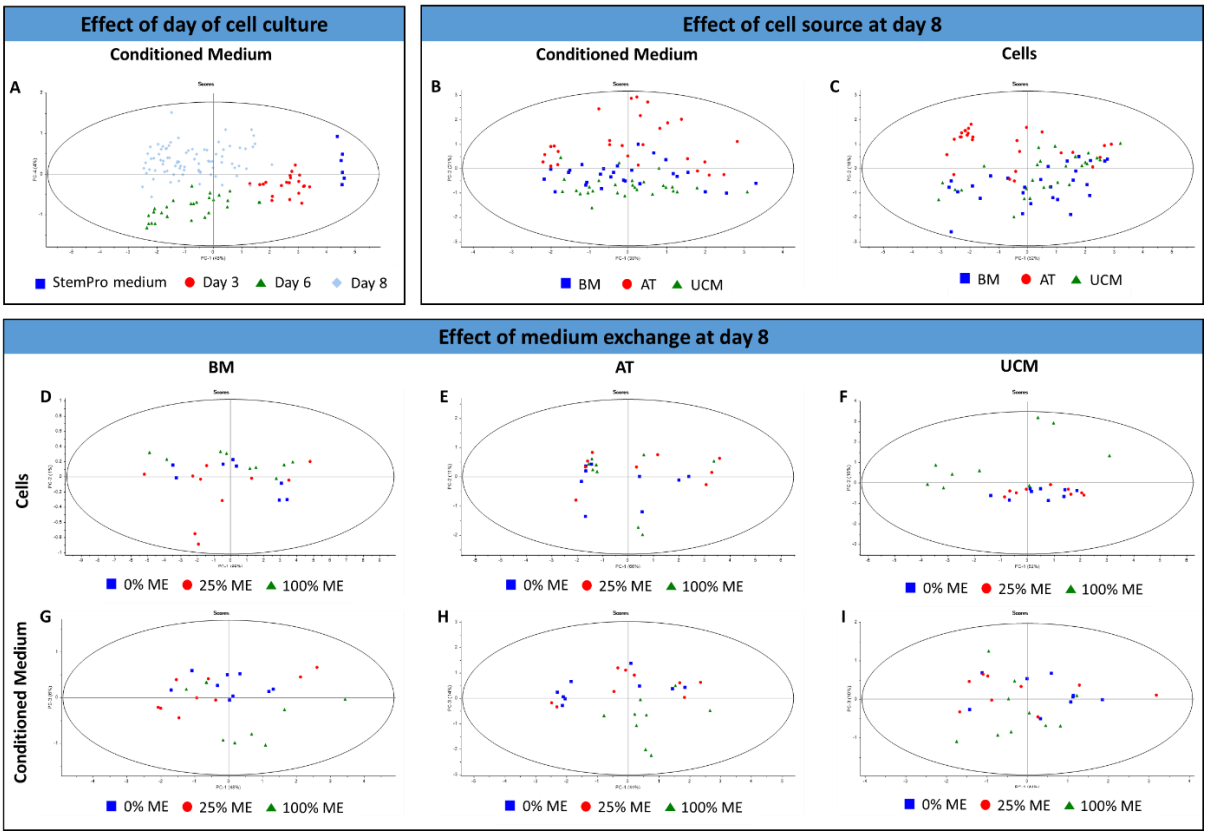

Figure 4.4- PCA scores plots for spectral data from conditioned medium and MSC retrieved at different time points during the time point experiment. PCA scores plots for spectral data from **(A)** conditioned medium and fresh medium *vs* day of culture (0, 3, 6 and 8); **(B)** conditioned medium and **(C)** cells *vs* MSC source (BM, AT and UCM) at day 8; **(D, E, F)** conditioned medium and **(G, H, I)** cells *vs* percentage of medium exchange (0%, 25% and 100%) for BM **(D, G)**, AT **(E, H)** and UCM **(F, I)**. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix; ME, medium exchange).

Analyzing the molecular profile of CM from day 8 of experiment for each percentage of medium exchange (**Figure 4.4G**, **Figure 4.4H** and **Figure 4.4I**), it is noticed an alteration in the molecular composition of the three cell sources when 100% of the medium was changed at day 6 (green triangles), less evident in the case of UCM MSC. In the case of the molecular profile from cell samples (**Figure 4.4D**, **Figure 4.4E** and **Figure 4.4F**), it was observed different molecular profiles of BM and UCM MSC also for 100% of ME (green triangles). This impact on the molecular composition for CM and cell samples once the medium was completely changed at day 6 of experiment, can be related with a more significant presence of vesicles added from the cultured medium, with a higher level of cellular communication (number of secreted EVs and other soluble factors higher than the internalized ones), with a higher viability and ”healthier“ state of the cells (due to higher availability of glucose and lower lactate amount), or with the higher cell number.

Of note that MSC-EV samples were not analyzed by FTIR spectroscopy as the PEG precipitation agent had a high signal, masking the signal of the EVs.

4.1.3. MSC-EV Quantification

The isolated MSC-EVs were quantified by NTA and the EV production profile for the three sources throughout the 8 days was analyzed (**Figure 4.5A**). To be used as control, a fresh sample of the medium employed in MSC culture was incubated in a T-Flask for 2 days (corresponding to the time of medium conditioning) under the same conditions of MSC culture and handled equally to quantify the particles present.

Since EVs are intercellular communication mediators, cells capture and internalize both particles and EVs already contained in the fresh culture medium, as well as the EVs produced and secreted by other cells in culture. In this manner, the optimization of EV production must be based on the maximization of EV production relatively to EV uptake. The constant production and uptake of EVs throughout culture time was considered in this work.

Considering that the amount of particles present in the fresh culture medium (7.39×10^{11} particles/mL) is added to the culture in the beginning of the experiment, and that it is the initial amount of particles in MSC culture, it seemed that the uptake of particles by cells was higher than the EV production until day 3, for the three cell sources (**Figure 4.5A**). Of notice, NTA provides the concentration of the particles present in the samples, however, the particles may or may not be EVs. UCM MSCs yielded the highest average EV concentration in the CM at day 3 ($(2.6 \pm 0.69) \times 10^{11}$ particles/mL). The average EV concentration at day 3 was lower and similar for BM and AT MSC (0.87×10^{11} and $(1.1 \pm 0.26) \times 10^{11}$ particles/mL, respectively; EV concentration of BM MSC at day 3 from one donor only). Furthermore, there is also a lower amount of cells in culture that could produce EVs.

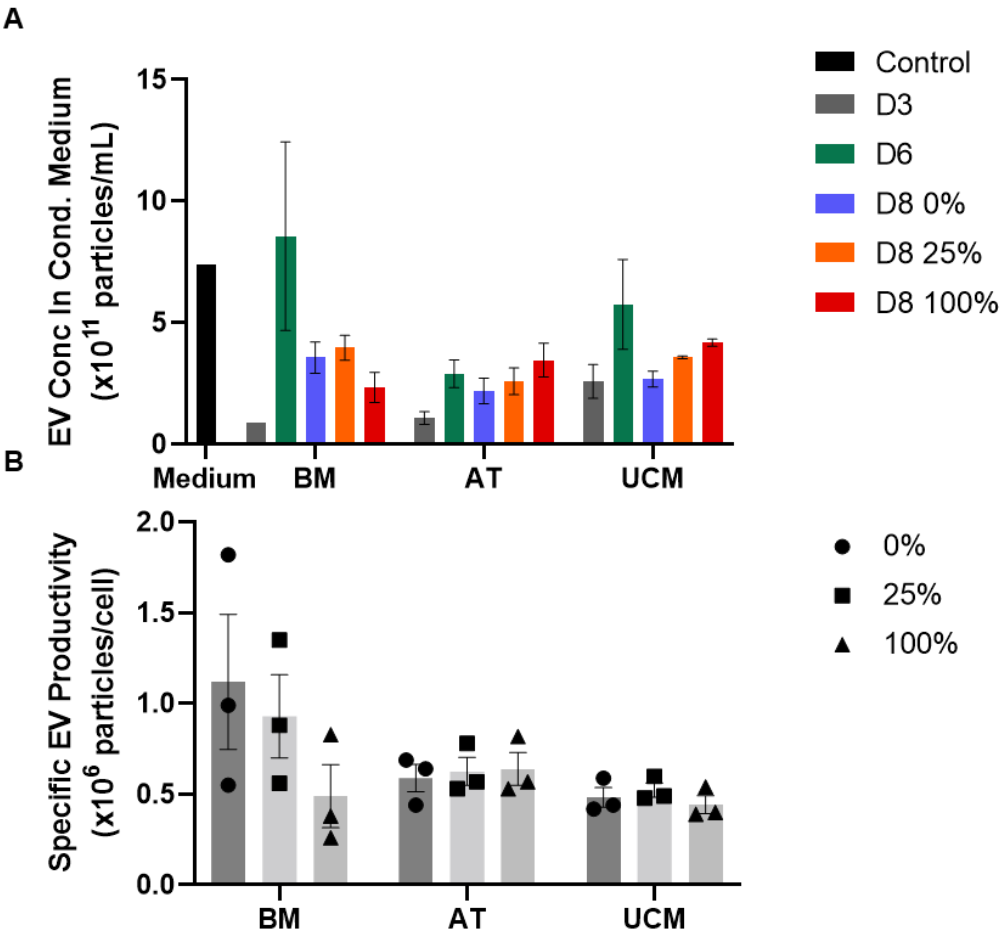

Figure 4.5- Influence of the percentage of culture medium exchange at day 6 on the final MSC-EV concentration at day 8. **(A)** EV concentration (x10^{11} particles/mL) in the conditioned medium retrieved at different time points from BM, AT, and UCM MSC cultures under static conditions. Samples of conditioned medium were harvested at day 3 (grey), 6 (green) and 8, and EVs were isolated with PEG solution. Fresh culture medium was used as control (black). Three different percentages of medium exchange were performed at day 6 (before medium conditioning stage), 0 % (blue), 25% (orange) and 100 % (red). MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1). **(B)** Specific EV productivity (x10^6 particles/cell) in the conditioned medium retrieved from BM, AT, and UCM MSC cultures in static system. MSC from three different donors were used for each tissue source and for each donor three different percentages of medium exchange were applied at day 6, 0 % (circle), 25% (square) and 100 % (triangle). Results are presented as mean ± SEM (n = 3). To reveal the variation across the measurements, these data are plotted as individual points, and the average of these measurements is also shown. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

At day 3, the medium was renewed completely and therefore, it is plausible to consider that the EVs existent in the CM at that time were removed and that culture medium-particles were introduced with the culture medium added. From day 3 until day 6, the uptake of particles and vesicles by the cells seemed lower or more balanced with the EV production (**Figure 4.5A**). The EV concentration in the CM was higher at day 6 comparing to day 3, and more similar to the particle concentration present in the culture medium, but this observation differed between sources. BM MSCs yielded the highest average EV concentration in the CM at day 6 ((8.6 ± 3.9) × 10^{11} particles/mL) but also the most heterogeneous among donors (n = 3), followed by UCM MSCs ((5.8 ± 1.8) × 10^{11} particles/mL) and then AT MSC ((2.9 ± 0.56) × 10^{11} particles/mL). At this stage, the cells were in exponential growth phase leading to high confluence at day 6 (around 80%), which means that there were increasingly more cells to uptake particles and EVs but also to produce it.

At day 6, three different percentages of ME were performed to investigate the influence in MSC-EV production. Therefore, there are three different possible analysis for the EV concentration in the CM at day 8. In the case of 0% ME, the EVs present in the CM at day 6 were not removed and neither particles from the culture medium were added. Therefore, it seems that for this condition, in the case of BM and UCM MSC, the uptake of EVs was higher than the production between day 6 and day 8, since the EV concentrations at day 8 ((3.6 ± 0.65) × 10^{11} particles/mL and (2.7 ± 0.33) × 10^{11} particles/mL, respectively) were lower than at day 6 (**Figure 4.5A**). For AT MSCs, EV concentration at day 8 ((2.2 ± 0.52) × 10^{11} particles/mL) was similar to day 6, which possibly indicates that the EV uptake and production balanced each other between these days. In the case of 25% ME, some of the EVs present in the CM at day 6 were removed and particles from the fresh culture medium were added. The EV concentration varied similarly to the condition of 0% ME (**Figure 4.5A**). For BM and UCM MSC the uptake of EVs seemed also higher than the production between day 6 and day 8 (EV concentrations at day 8, (4.0 ± 0.51) × 10^{11} particles/mL and (3.6 ± 0.060) × 10^{11} particles/mL, respectively, were lower than at day 6) and for AT MSC seemed that balanced each other (EVs concentration of (2.6 ± 0.55) × 10^{11} particles/mL at day 8). It is important to remark that the discrepancy on EV concentration at day 6 was relatively high between the different donors from BM and UCM, and thus, the differences in the averages of EV concentration between day 6 and 8 observed for the conditions of 0% and 25% of ME for these sources could not be statistically significant. These observed heterogeneities emphasize the importance of testing MSC from multiple donors of each cell source to assess for intrinsic biological variability.

Finally, when 100% of the medium was changed at day 6, it is expected that the EVs present in CM at day 6 were totally removed and particles from the fresh culture medium were added. Here, it is necessary to compare again the EV concentration at day 8 with particles concentration from the fresh medium added. In this manner, the uptake of EVs seemed also to surpass the production between day 6 and day 8 for the three sources, more sharply for BM MSC (**Figure 4.5A**). The EV concentration at day 8 ((2.3 ± 0.61) × 10^{11} particles/mL, (3.5 ± 0.69) × 10^{11} particles/mL and (4.2 ± 0.15) × 10^{11} particles/mL, for BM, AT and UCM MSC respectively) were lower than the particle concentration in the culture medium added.

These three conditions of ME were also compared in terms of EV productivity (**Figure 4.5B**), that in the end is the factor that matters for the optimization of EV production per cell, the specific EV productivity. For that, EV concentration in the CM at the end of experiment (day 8) was divided by the cell concentration at that time. BM MSC donors showed more heterogenous specific EV productivity between the three ME conditions (between (0.49 ± 0.17) × 10^{4} particles/cell and (1.1 ± 0.37) × 10^{4} particles/cell) which is indicative that donor variability could be reflected in the EV productivity. AT and UCM MSC specific EV productivity showed similar values between ME conditions (between (0.59 ± 0.070) × 10^{4} particles/cell and (0.64 ± 0.090) × 10^{4} particles/cell; and between (0.44 ± 0.040) × 10^{4} particles/cell and (0.52 ± 0.040) × 10^{4} particles/cell, respectively).

Globally, the total renovation of the culture medium at day 6 (100 % of ME) seemed to allow the cells to grow healthier, leading to a higher cell number at the end of the experiment (**Figure 4.1**), and to allow an increase in EV concentration in the CM for AT and UCM MSCs (**Figure 4.5A**). However,

besides these increases could not be statistically significant, this percentage of ME did not seem to have a positive effect on EV productivity comparing to the other percentages (**Figure 4.5B**). BM MSCs showed the lowest EV productivity when applied 100% ME, which can be explain by the lowest EV concentration in the CM and to the higher cellular uptake of particles. Additionally, 100% ME showed no significative variation for AT and UCM MSCs EV productivity comparing to the other ME conditions. Therefore, the higher number of cells at the end of experiment did not reflect necessarily in a higher EV concentration in the CM (as seen for BM MSCs) and a higher EV productivity. Furthermore, when the medium was fully removed at day 6, it is more likely to expect a higher amount of possible contaminant particles from the culture medium in the final EVs samples. Altogether, the percentage of ME at day 6 seemed not to have significant influence on EV productivity.

4.1.4. Purity assessment

The EVs were isolated from MSC CM using a method based on precipitation with a PEG solution, one of the most popular methods for EV isolation due to its simplicity, quickness, and for not requiring special equipment (just a regular centrifuge at low speed). This isolation method allows to process a large number of samples simultaneously, does not deform EVs (as the centrifugation speed is low) and requires no additional and expensive equipment for EV isolation. However, this approach is considered to result in low purity as the precipitation agent co-isolates contaminants considered non-vesicular materials, including diverse types of proteins which indeed can be co-precipitated in the form of insoluble aggregates. Additionally, the molecules of biopolymers can also integrate the EV fraction and possibly interfere with further analysis of the sample (Konoshenko et al. 2018).

Therefore, to investigate the purity of the EV samples obtained, the protein to particle ratio (PPR) was determined by dividing the total protein concentration (determined through microBCA protein assay) (**Supplementary Material 7.4**) by the EV concentration of the same sample (determined by NTA, **Figure 4.5A**) (Webber and Clayton 2013). The lower the PPR values, the lower is the amount of co-isolated protein contaminants after EV isolation and higher the sample purity. The PPR values from the EV samples (**Figure 4.6A**) were heterogeneous between days of sample harvesting and conditions of ME. BM MSC-EV sample from day 3 presented the higher PPR (9 fg protein/ EV particle), which means the lower purity. While UCM MSC EV sample from day 6 presented the lower PPR (2.2 ± 0.73 fg protein/EV particle), which means the higher purity. Comparing these values with the PPR from the unpurified CM that were much higher (**Supplementary Material 7.6**), it is possible to observe that the purity was largely increased after the EV isolation and that the majority of non-vesicular materials were removed from the CM samples. The values of concentration factor (**Figure 4.6B**), calculated dividing the EV concentration (determined by NTA) from EV samples (**Figure 4.5A**) by the EV concentration from CM samples (**Supplementary Material 7.5**), are also in agreement with this, ranging between 7.89 and 40.9 ± 17.2 and showing that besides the variability in the concentration efficiency and in the EV isolation, the EV samples were concentrated to a high extent after the isolation with PEG solution.

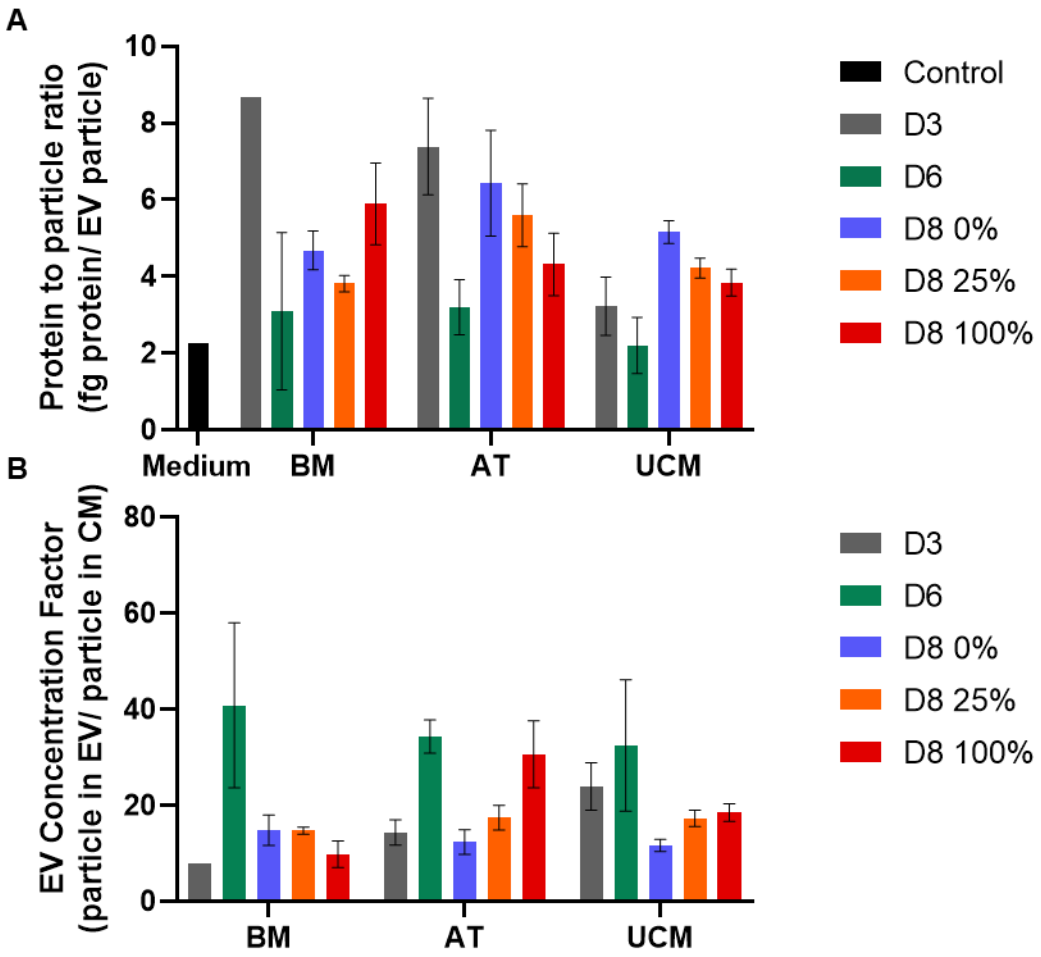

Figure 4.6- (A) Purity assessment through the protein to particle ratios (PPR) (fg protein/ EV particle) of EV samples obtained throughout BM, AT and UCM MSC culture in static system, at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange). MSC from three different donors were used for each tissue source. Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1). **(B)** EVs concentration factor (particle in EV/particle in CM) after isolation with PEG solution from samples obtained throughout BM, AT and UCM MSC culture in static system, at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange). MSC from three different donors were used for each tissue source. Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1). BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

However, although it was observed a significant increase in purity and concentration in EV samples, it is still possible that the EV samples presented considerable contaminants. Attending the purity classification proposed by Webber and Clayton in one of the few studies with a detailed purity analysis of EV samples using the PPR values (Webber and Clayton 2013), the EV samples obtained in this experiment were still not pure. According to this purity classification, PPR lower than 0.03 fg protein/EV particle is considered high purity, PPR between 0.05 and 0.5 is low purity and PPR higher than 0.67 is impure. In this experiment, all EV samples showed PPR values higher than 0.67 (**Figure 4.6A**) and thus can be considered impure according to Webber and Clayton purity classification of EV samples. Nevertheless, Webber and Clayton evaluated the purity of EVs isolated by other method (ultracentrifugation), from different cell type (several cancer cell lines) and using different culture medium, which could influence these purity classification levels.

If the EV samples would be used as therapeutic product, a second method of EV purification should be applied in order to increase the purity of the EV samples and to remove the PEG precipitation agent.

4.1.5. Size distribution

NTA was also used to determine the size distribution of MSC-EVs. MSC-EV samples from the three sources showed no visible variation in size distribution profile between each other and throughout the experiment (**Figure 4.7A**), except in the case of EV samples from day 3 of the experiment, that showed a more heterogenous size distribution profile and a more notorious presence of vesicles with higher sizes, for the three different sources. Furthermore, MSC-EV samples were mostly enriched in small EVs (<200 nm) (**Figure 4.7B**). Curiously, the sample of fresh medium used as control showed a more heterogenous size distribution profile and higher mode size of particles (**Figure 4.7A** and **Figure 4.7B**). The presence of particles derived from the culture medium in the EV samples from day 3 could explain the more heterogenous size distribution profile and the presence of particles with higher sizes.

To produce MSC-EVs for therapeutic applications, the ideal would be the use of a culture medium that do not contain EVs or EV-like particles that will be co-isolated with the MSC derived EVs. Culture medium supplements such as FBS and hPL have a large amount of protein and vesicle contents, presenting an additional challenge for their use in EV manufacturing. These components are prone to be co-isolated with the EV fraction, thus contaminating the end product. There are already evidences that these exogenous EVs are likely to be safe, and could even contribute to therapeutic effects directly or acting as co-factors, as seen for hPL vesicles (Witwer et al. 2019). However, exogenous EVs or EV-like particles could still conceivably dilute or block some effects of MSC-EVs.

For this work, StemPro® MSC SFM XenoFree medium was chosen for being well-defined and xeno/serum-free and thus considered a better option for therapeutic applications than, for example, FBS or hPL supplemented media. However, as shown by NTA analysis (**Figure 4.5A**- black bar), this medium also present particles that could be interfering in MSC-EV quantification. To avoid this interference and the contamination of MSC-EV fractions, the StemPro® supplement could have been removed at the end of the MSC expansion period and StemPro® supplement-free medium used for the medium conditioning period. As seen when the medium was not change (0% ME), that the number of cells was lower at the end of experiment comparing to the other conditions of ME (**Figure 4.1**), the elimination of the supplement can be considered a possible stress factor for cell culture and could induce a different molecular profile to the MSC CM and EVs. Nevertheless, in the future, a comparison between supplemented and non-supplemented culture medium, as well as comparing with supplemented filtered medium (to ensure the removal of the culture medium particles) could be considered.

The fact that the particles present in the StemPro® MSC SFM XenoFree medium used for MSC expansion and medium conditioning could have interfered in the quantification and characterization of EVs produced by MSCs in the time point experiment, led to the suggestion of using filtered medium to try to remove those contaminant particles. Since the NTA analysis of a fresh sample of StemPro® MSC SFM XenoFree medium identified that most of the particles present had size above 100 nm (**Figure 4.7**), it was decided to filter the medium with 0.1 µm filter to try to remove those particles and study MSC-EV production.

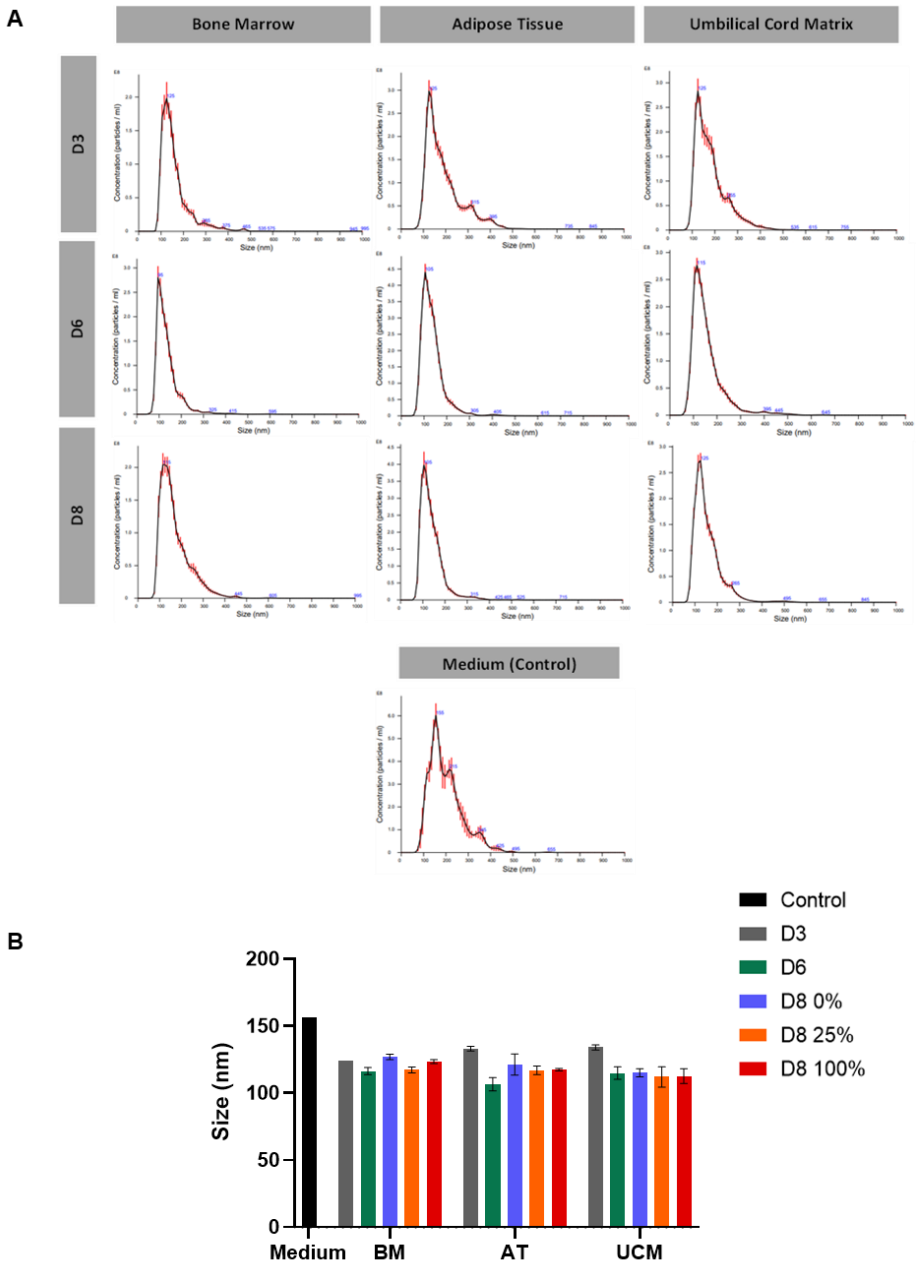

Figure 4.7- (A) Representative size (in nm) distribution curves of EV samples obtained at day 3, 6 and 8 of BM, AT, and UCM MSC cultures in static system, and from fresh medium StemPro® MSC SFM XenoFree used in the experiment. **(B)** Mode sizes (nm) of EV samples obtained at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange) from BM, AT, and UCM MSC cultures in static systems, and from fresh medium StemPro® MSC SFM XenoFree used in the experiment (black). Results are presented as mean ± SEM. MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

4.2. StemPro *vs* filtered StemPro medium experiment

4.2.1. Influence of MSC culture conditions on particles from fresh culture medium

In one of the few studies regarding heat stability of EVs, Schulz and his group exposed B lymphoblastoid cell-derived EVs and also outer membrane vesicles derived from myxobacterial species *Sorangiineae* to 37 °C, 50 °C, 70 °C and 100 °C for 1 h, 6 h and 24 h, and also autoclaved them. Interestingly, they observed minor alterations in particle concentration, size and in protein concentration after those exposures, especially at 37 °C (Schulz et al. 2020).

In order to understand whether the particles present in the StemPro® MSC SFM medium could remain in culture (at 37 ºC) for more extended periods and then possibly interfere in the quantification and characterization of MSC-EVs, a T75-flask with fresh StemPro® MSC SFM XenoFree medium (without cells) was maintained for 7 days in the same conditions of MSC culture (37ºC, 5% CO_2 and >95% humidity). During this time, samples of medium were retrieved at days 1, 3 and 7 to investigate the evolution of particle concentration in the medium. A sample of fresh medium was also analyzed and considered as day 0. Particles were isolated from the samples using the precipitation method with PEG solution and the particles concentration in each fraction was obtained using NTA. Preliminary results indicate that the particles concentration in the medium decreased 70% throughout the 7 days under the conditions imposed (from $3.8 \pm 0.20 \times 10^{10}$ particles/mL to $1.2 \pm 0.040 \times 10^{10}$ particles/mL) (**Figure 4.8**). This result indicates that there could be particle degradation along culture time caused by the temperature of incubation and/or particle adhesion to the plastic of the T-flasks.

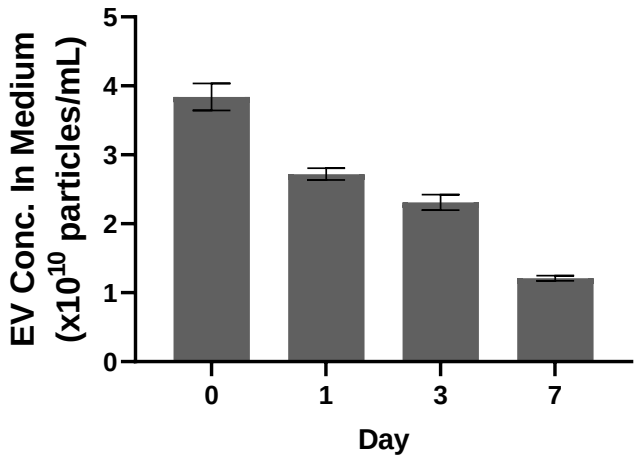

Figure 4.8- Variation of EV concentration ($x10^{10}$ particles/mL) in StemPro® MSC SFM XenoFree culture medium incubated at the same conditions of MSC culture (37ºC, 5% CO2 and >95% humidity) throughout 7 days. Results are presented as mean ± SD.

Although these results showed that particles from the medium could be degraded during MSC culture, it was still observed that a large amount of particles remain in the medium after 7 days of incubation at 37ºC (1.21×10^{10} particles/mL). Therefore, even if the medium is not changed throughout MSC expansion and medium conditioning, the hypothesis that the particles present in the medium could be interfering with the quantification and characterization of EVs produced by MSCs is valid. This concerning fact consolidated the necessity of trying to remove the particles present in the medium to avoid contamination of EV samples.

4.2.2. Influence of culture medium filtration on MSC culture and EV production

To reduce the number of EVs present in the culture medium, StemPro® MSC SFM XenoFree medium was filtered with 0.1 µm filter (SPf) prior to MSC culture. To study the impact of SPf on MSC growth and EV production, one donor of BM MSC was expanded with SP and SPf during 5 days, considered the necessary time to achieve around 80% cell confluence and do not impose stress on cells due to the lack of space to attach and grow during medium conditioning. The next 2 days were considered for medium conditioning. The medium was totally changed at day 3 to support MSC expansion and two percentages of ME at day 5 were selected to be studied again, 0 and 100%. After 7 days, EVs were isolated from the CM and the cells counted.

It was observed that the total cell number was slightly lower for the conditions using SPf, 1.48 × 10^6 cells and 1.79 × 10^6 cells for 0% ME of SPf and SP, respectively, and 2.26 × 10^6 cells and 3.19 × 10^6 cells for 100% ME of SPf and SP, respectively (**Figure 4.9**). Even so, the use of filtered medium seemed to be feasible for MSC expansion.

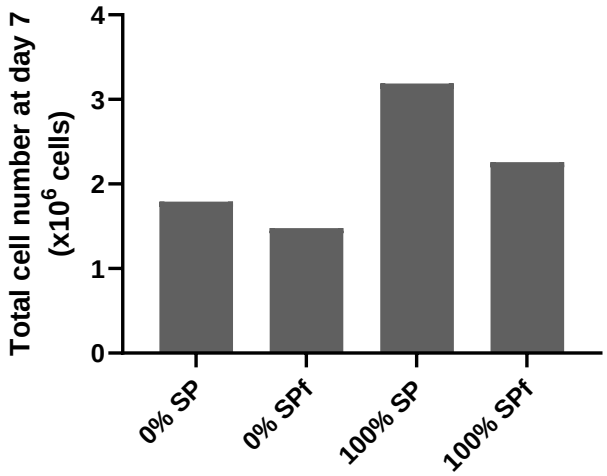

Figure 4.9- Total cell number (x10^6 cell) counted at day 7, the last day of the experiment, after 5 days of MSC expansion followed by 2 days of medium conditioning, for BM MSC (n=1) cultured in static system. Two percentages of medium exchange were applied at day 5 (0 and 100%) for StemPro and filtered StemPro. SP-StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

In terms of EV concentration in the CM (determined using NTA), the preliminary results showed that the variation between conditions was similar (**Figure 4.10A**). When the SPf was used, EV concentrations were lower comparing to SP, more sharply in the case of 0% ME (from (4.9 ± 0.11) x 10^{10} particles/mL to (2.9 ± 0.090) x 10^{10} particles/mL) than 100% ME (from (3.1 ± 0.090) x 10^{10} particles/mL to (2.4 ± 0.080) x 10^{10} particles/mL), although it did not seem significant. These differences in EV concentration could indicate that the SPf medium added less particles to the culture than the SP medium. Comparing the conditions of 0% and 100% ME (with or without medium filtration), EV concentration was higher when the medium was not changed at day 5.

In the case of EV productivity (**Figure 4.10B**), it seemed to be higher when the medium was not changed at day 5 (filtered medium or not), as seen before for BM MSC in the time point experiment (**Figure 4.5B**). Although it seemed that cells grown healthier when the medium was completely changed

at day 5 (**Figure 4.9**), leading to a higher cell number at the end of the experiment, it had not positively influence in EV productivity.

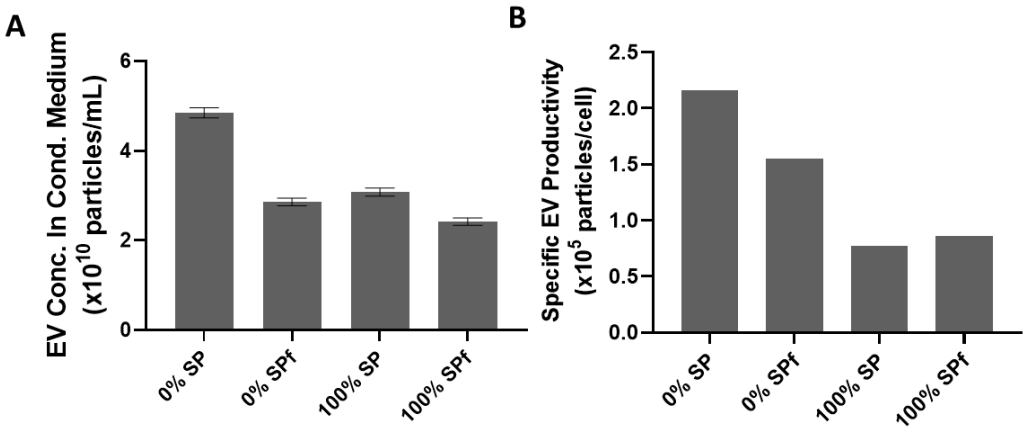

Figure 4.10- Comparing the influence of different percentages of medium exchange when using StemPro and filtered StemPro in MSC-EV production, using BM MSC in static system (n=1). **(A)**- EV concentration (x10^{10} particles/mL) in the cell culture conditioned medium from BM MSC in the end of the 7 days of culture. Results are presented as mean ± SD. **(B)**- Specific EV productivity (x10^5 particles/cell) in the cell culture conditioned medium from BM MSC culture in static system. SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

The size of the EVs after isolation from the CM with PEG solution were similar between the different conditions, slightly higher than 100 nm, and also similar to the size of EVs present in the unpurified samples of cell culture CM (**Figure 4.11**).

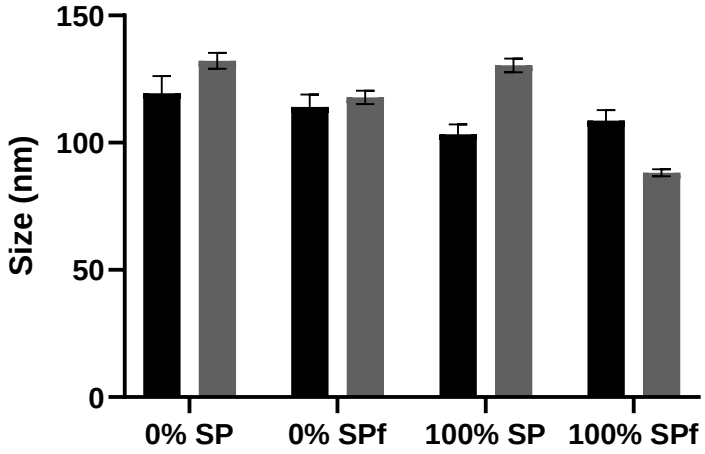

Figure 4.11- Mode sizes (nm) from EV samples (black) and unpurified cell culture CM samples (grey) from BM MSC culture in static systems. Results are presented as mean ± SD. SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

In relation to purity of EV samples assessed through the PPR, it was observed a significant increase in purity after isolation with PEG solution, since the PPR values from EV samples (black bars) decreased significantly comparing to unpurified CM samples (grey bars) (**Figure 4.12A**). According to this, it was also observed that, in general, the EV concentration increased (**Figure 4.12C**) and protein concentration decreased significantly (**Figure 4.12D**) in EVs samples (black bars) comparing to

unpurified CM samples (grey bars), reflecting the values of concentration factor for EVs (black bars-higher than 1) and protein (gray bars-lower than 1) (**Figure 4.12B**). However, the PPR values from EVs samples were still elevated (between 11.79 fg protein/ EV particle and 22.7 fg protein/ EV particle) and higher than in case of samples from time point experiment (PPR values lower than 10 for all samples-**Figure 4.6A**), indicating that samples were still impure. The higher protein contamination could be due to lower yield of EV isolation method used, possibly a consequence of incomplete removal of supernatant after the centrifugation step (and therefore lower removal of protein) and of higher dilution of the EVs in the final resuspension with DNase/RNase-Free PBS 1x.

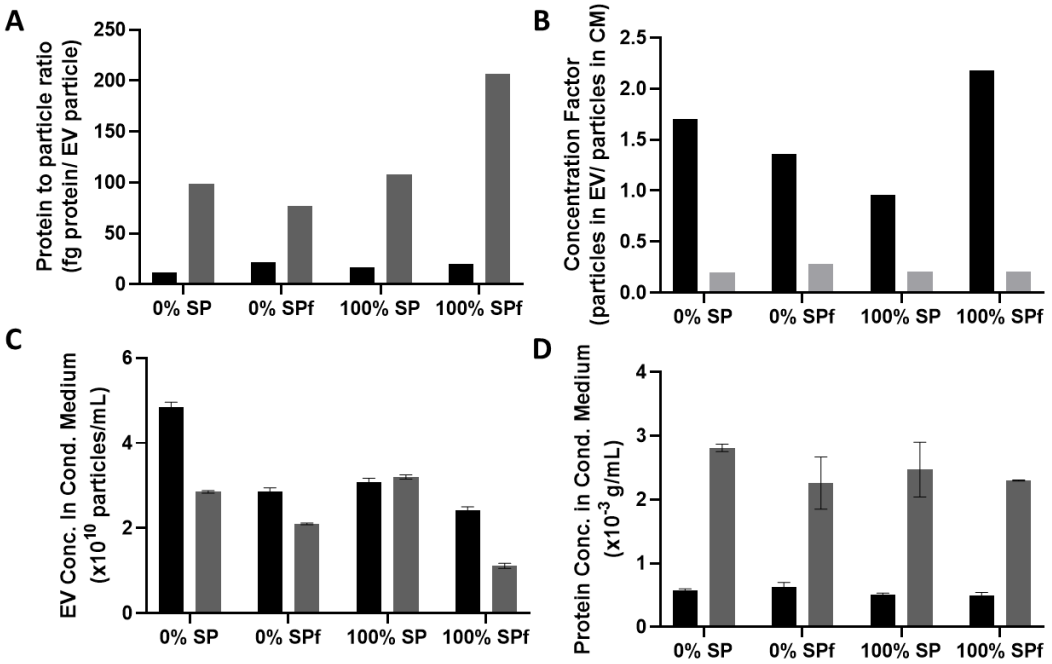

Figure 4.12- Purity assessment of EV samples from BM MSC culture in static systems with different conditions of medium exchange, 0 and 100%, using StemPro® MSC SFM XenoFree and filtered StemPro® MSC SFM XenoFree. **(A)-** Protein to particle ratios (PPR) (fg protein/EV particle) of EV samples (black) and unpurified CM (grey). **(B)-** EV (black) and protein (gray) concentration factor (particle in EV/particle in CM) from EV samples obtained after isolation with PEG solution. **(C)-** EV concentration (x10^{10} particles/mL) in EV samples (black) and unpurified CM samples (grey). Results are presented as mean ± SD. **(D)-** Protein concentration (x10^{-3} g/mL) in EV samples (black) and unpurified CM (grey). Results are presented as mean ± SD. CM- unpurified CM samples; SP-StemPro® MSC SFM XenoFree; SPf- filtered StemPro® MSC SFM XenoFree.

This experiment was designed to try to understand if the use of filtered medium during the study of MSC-EV production could avoid the addition of particles contained in the culture medium that contaminate the MSC-EV fractions. Overall, these preliminary results showed that the use of filtered medium did not significantly affect MSC expansion and confirmed the idea that exchange 100% of the culture medium before medium conditioning did not have a positive effect on EV productivity when compared with 0% ME, also considering that it implies a higher spent of medium. However, without data from samples throughout MSC expansion and before medium conditioning, it is difficult to understand the profile of uptake/production of EVs and to be sure that the filtration of the medium in fact removed enough particles from the medium to avoid the contamination of MSC-EV samples and the interference in MSC-EV quantification and characterization.

To minimize the addition of EVs present in the fresh culture medium, the same experiment was carried out, without medium changes. The goal was to try to better understand the profile of uptake/production of EVs by MSCs without having the influence of particles added once the medium is changed. Another donor of BM was selected and MSC-EV production was studied again for 7 days. Samples of CM were harvested at day 1, 3, 5 and 7 for EV isolation and further characterization. Samples of fresh SPf and SP culture media were also processed and considered as day 0.

Firstly, comparing the curves of size distribution obtained from NTA for unpurified samples of fresh media (SP and SPf) before EV isolation (**Figure 4.13**- top curves), it is possible to observe the impact of the filtration with 0.1 µm filter. After filtration, the mean size of particles presented in the medium decreased from 134 ± 1.2 nm to 116.2 ± 1.2 nm, and the mode size from 104.2 ± 3.1 nm to 92.7 ± 2.3 nm. However, particles with size higher than 100 nm were still observed in the filtered medium and, although the particle concentration decreases in relation to the normal medium ((1.9 ± 0.040) × 10^{10} particles/mL), it is also observed a high particle concentration in the filtered medium ((1.6 ± 0.030) × 10^{10} particles/mL) (**Figure 4.14A**- Day 0). Therefore, it seems that the filtered medium could still add particles to the culture and possibly contaminate the MSC-EV samples. Curiously, the effect of filtration is not observed analyzing the curves of size distribution from the purified samples (**Figure 4.13**- bottom curves). After the isolation of EVs from the media using the precipitation method, could have occurred particle aggregation that caused an increase in the particle size detected using NTA (mode sizes of 182.1 ± 5.5 nm for SP and 187.8 ± 7.4 nm for SPf).

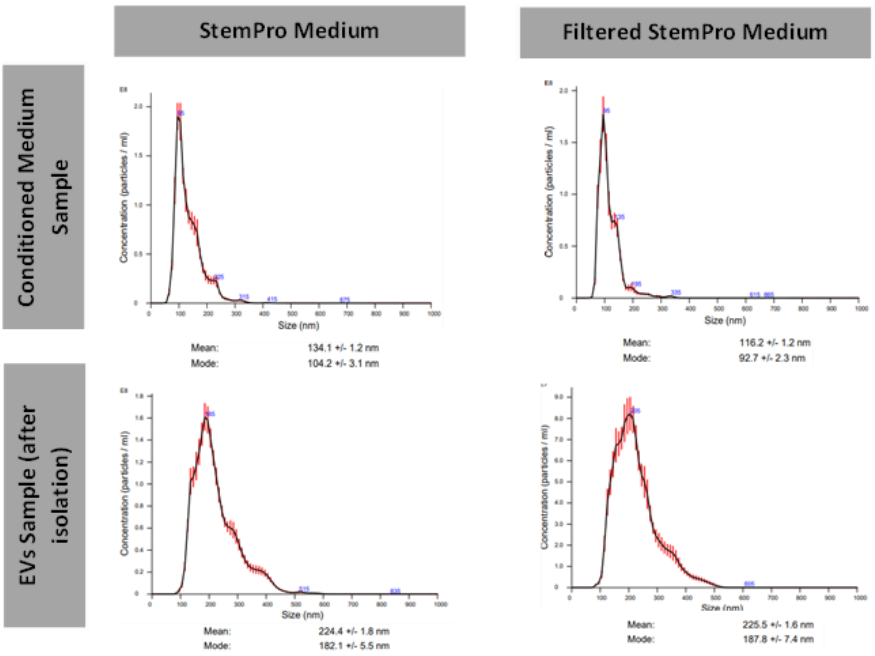

Figure 4.13- Size distribution curves and respective mean and mode sizes (± SD) of unpurified samples and EV samples (after isolation) of fresh StemPro® MSC SFM XenoFree medium and fresh StemPro® MSC SFM XenoFree medium filtered with 0.1 µm filter obtained using NTA.

Comparing the variation of EV concentration throughout the experiment when using SP and SPf (Figure 4**.14**A), it is observed that there were not considerable differences. The choice for the filtration of the medium with 0.1 µm filters was a question of filters availability with the addition of possibly removing the particles from the medium that seemed to have a size higher than 100 nm. However, as observed by the analysis of fresh media samples (**Figure 4.13** and **Figure 4.14A**- Day 0), that filtration seemed to not remove a significant amount of vesicles from the medium (EV concentration decreased only 16%, from (1.9 ± 0.040) × 10^{10} particles/mL to (1.6 ± 0.030) × 10^{10} particles/mL), it also had no significant influence throughout culture (**Figure 4.14A**). The total cell number at the end of experiment was very similar when using SP and SPf (**Figure 4.14B**), 2.64 × 10^6 and 2.82 × 10^6, respectively. The specific EV productivity (**Figure 4.14C**) was slightly higher when SPf was used, 4.73 × 10^6 particles/cell compared to 3.49 × 10^6 particles/cell when was used SP. Since the ideal condition for MSC-EV production would be the use of a medium without particles, in the future, filters with lower pore size or alternative methods (ultracentrifugation) could be considered for a more efficient particle removal and to remove particles below 0.1 µm.

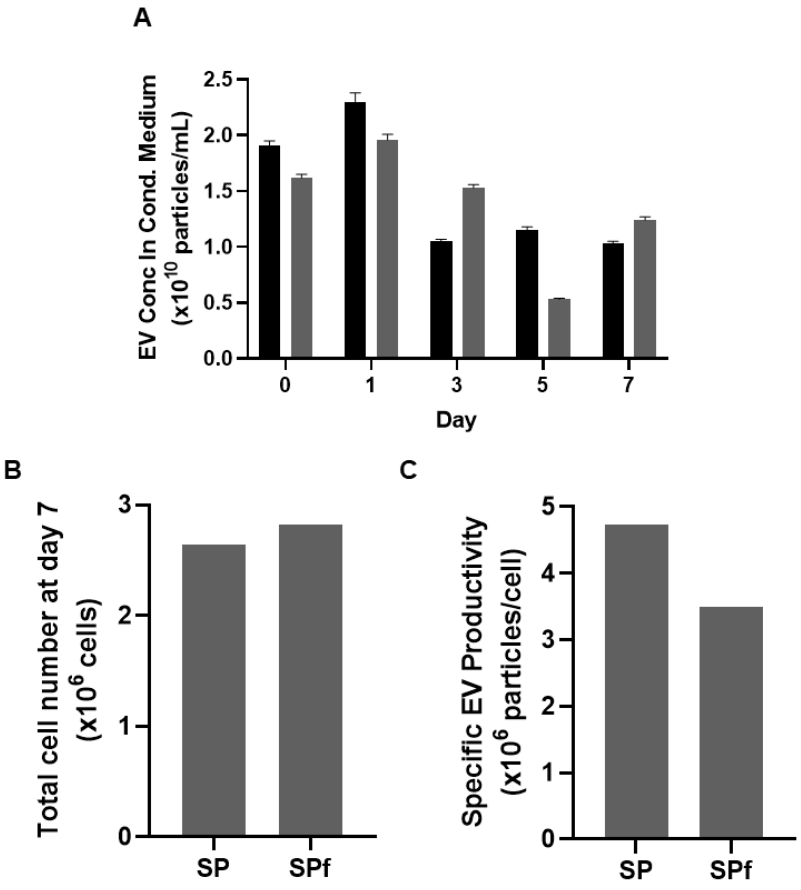

Figure 4.14- Study of MSC-EV production and profile of uptake/production of EVs by MSCs using StemPro and filtered StemPro medium in BM MSC culture in static system (n=1), without medium exchanges throughout 7 days of experiment. **(A)**- EV concentration (x10^{10} particles/mL) in the cell culture conditioned medium from BM MSC at days 1, 3, 5 and 7 of culture, when using StemPro medium (black bars) and filtered StemPro medium (grey bars). Fresh samples of the media were considered as day 0. Results are presented as mean ± SD. **(B)**- Total cell number (x10^6 cell) counted at day 7, the last day of the experiment, when using StemPro and filtered StemPro medium. **(C)-** Specific EV productivity (x10^6 particles/cell) in the cell culture conditioned medium. SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

In relation to the understanding of EV uptake/production profile throughout the experiment, the preliminary (n=1) results seem to indicate that the uptake of EVs was increasing in relation to production during MSC expansion (from the beginning until day 5), since the EV concentration seemed to decrease for both CM (**Figure 4.14A**). This is in agreement with the necessities of MSC growth. After cells reach 80% confluence (around day 5), EV concentration seemed to maintain identical values which could mean that EV uptake and production balanced each other. After day 5, there is still cell growth although to a lower extent compared to day 2 to 5 of the exponential phase. Nevertheless, it is expected the uptake of EVs by cells. Furthermore, during this period there are more cells to uptake EVs. However, there is also more cells to produce EVs. Therefore, it seems that the increased EV uptake is balanced by the increased EV secretion.

Without ME throughout 7 days, it is possible to assume that particles added from the medium in the beginning of the experiment were totally consumed during the first 5 days of culture, not causing a significative contamination of EV samples. However, it is difficult to be sure of that and thus, the use of a medium without particles seems to be the best option. On other hand, proving that particles from the medium used are not toxic and perhaps additionally beneficial for therapeutic applications, as reported before for example for hPL supplement (Witwer et al. 2019), the total removal of the particles from the medium could not be extremely necessary.

4.3. Influence of hypoxia and temperature in MSC-EV production

Previous studies have investigated EV production when different cell types (including MSC) were cultured under hypoxic conditions (ranging from 1% to 10 % O_2, compared to controls) suggesting an increase in EV secretion (Salomon et al. 2013; Sung et al. 2019; Dong et al. 2021). Nevertheless, other studies did not observed significant differences between EV concentration under normoxic and hypoxic conditions (Almeria et al. 2019; Andrade et al. 2021). Regarding temperature, previous studies using Chinese hamster ovary cells reported improved specific protein productivity when cultured at lower temperatures than 37ºC (ranging from 30ºC to 33ºC) (Kaufmann et al. 1999; Fogolín et al. 2004; Sung et al. 2004). Despite the different cell type and product, we hypothesized if lowering the temperature could improve MSC-EV production.

Therefore, to study the impact of these culture parameters, the influence of oxygen tension (normoxia *vs* hypoxia 5% O_2) and temperature during the conditioning phase (37ºC *vs* 33ºC) on MSC-EV production was studied using SP and SPf media.

It is necessary to remark that these conditions were only studied for one donor of BM in one isolated experiment and other donors need to be tested, and perhaps other cell sources, to reach an agreement of values and confirm any results presented here.

MSC were expanded with SP or SPf, under normoxic and hypoxic conditions for 7 days at 37ºC. At day 5, half of the cells cultured under normoxic conditions were transfer to another incubator operating under normoxic conditions at 33ºC. Samples of CM were also harvested throughout the experiment, at day 1, 3, 5 and 7, for EV isolation and further characterization. Although the T-flasks from conditions of normoxia at 37ºC and 33ºC (either using SP or SPf media) were at the same conditions during MSC expansion, EV concentration from both flasks were considered.

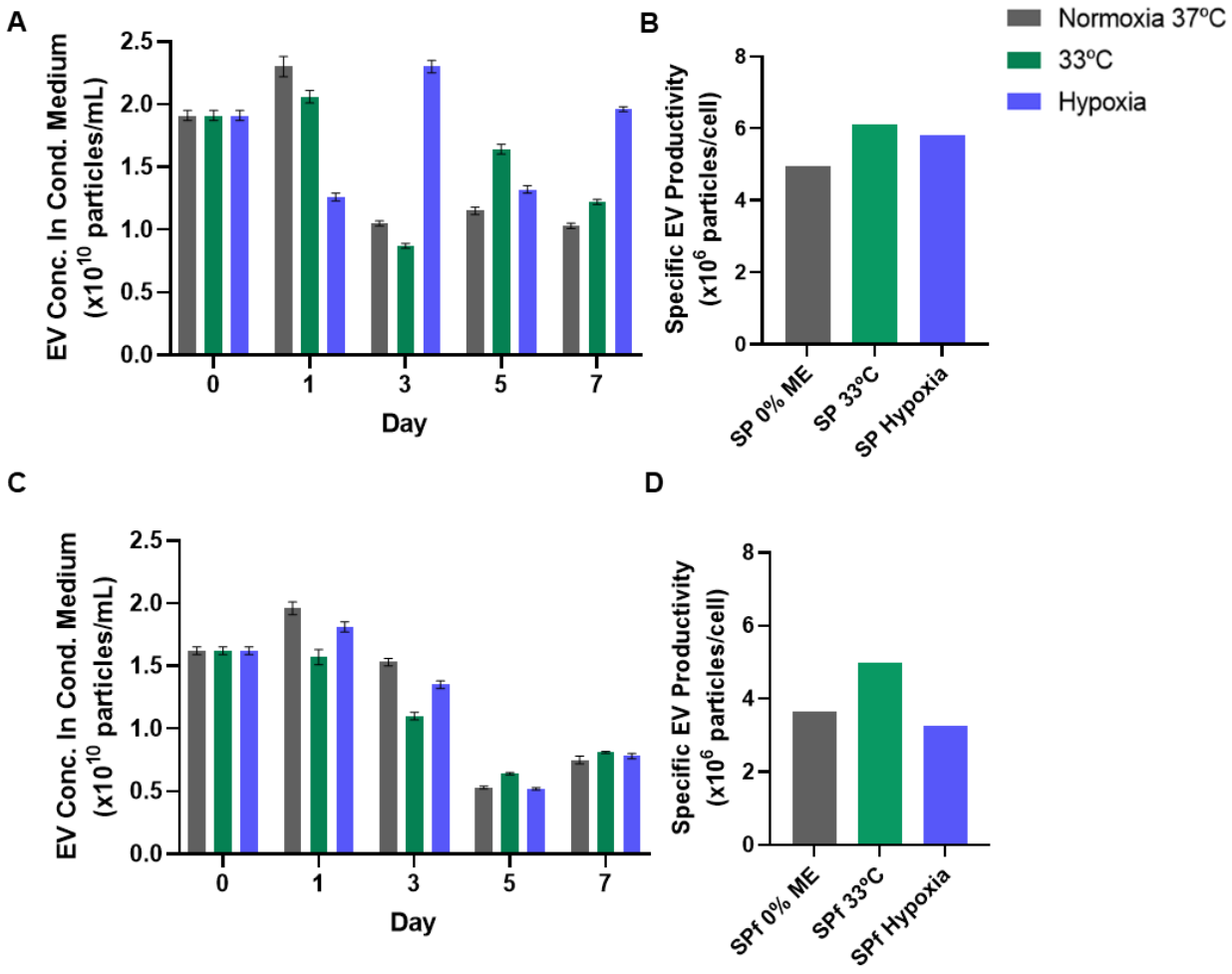

Figure 4.15- Comparing different conditions (oxygen tension and temperature) and their influence in MSC-EV production using BM MSCs (n=1) cultured in static system. **(A) and (C)-** EV concentration (x10^{10} particles/mL) in the cell culture conditioned medium when using StemPro medium and filtered StemPro medium, respectively, for the conditions of normoxia at 37ºC (grey), 33ºC during medium conditioning (green), and hypoxia (5% O_2) during MSC expansion and medium conditioning (blue). Samples of conditioned medium were harvested at day 1, 3, 5 and 7, and EVs were isolated with PEG solution. A fresh sample of each medium was considered as day 0. Results are presented as mean ± SD**. (B) and (D)-** Specific EV productivity (x10^6 particles/cell) in the cell culture conditioned medium when using StemPro medium and filtered StemPro medium, respectively, for the conditions of normoxia at 37ºC (grey), 33ºC during medium conditioning (green), and hypoxia (5% O_2) during MSC expansion and medium conditioning (blue). SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

The analyses of the preliminary results of EV concentration in the CM determined by NTA, indicate that hypoxia seemed to have influenced more the EV uptake/production during MSC expansion with SP (**Figure 4.15A**- blue bars) when compared with SPf culture medium (**Figure 4.15C-** blue bars). Indeed, when the SPf medium was used (**Figure 4.15C**), the concentration profile of EVs derived from MSC expanded under hypoxic conditions (blue bars) was identical to the one obtained under normoxic conditions at 37ºC (grey bars). Regarding the influence of the temperature during the conditioning phase (from day 5 to 7), no significant differences were observed between conditions.

Concerning specific EV productivity, the values obtained for normoxic (33ºC and 37ºC) and hypoxic conditions are consistently higher when SP culture medium was used (**Figure 4.15B** and **Figure 4.15D**). Specific EV productivity values of 4.9 × 10^6 and 3.7 × 10^6 particles/cell for normoxia at 37ºC and 6.1 × 10^6 and 5.0 × 10^6 particles/cell for normoxia at 33ºC were obtained for SP and SPf culture medium,

respectively. Regarding oxygen concentration, specific EV productivity values of 5.8×10^6 and 3.3×10^6 particles/cell were obtained for SP and SPf culture medium, respectively.

Of notice, the higher specific EV productivity at day 7, observed for the cultures maintained at 33ºC during the conditioning phase could be related with the lower cell number observed in this condition, when compared with the MSC expanded under hypoxia or normoxia at 37ºC (**Figure 4.16**). The total number of MSC obtained at day 7 (**Figure 4.16**) corroborate the previous experiments as no major differences were observed between the two culture media tested.

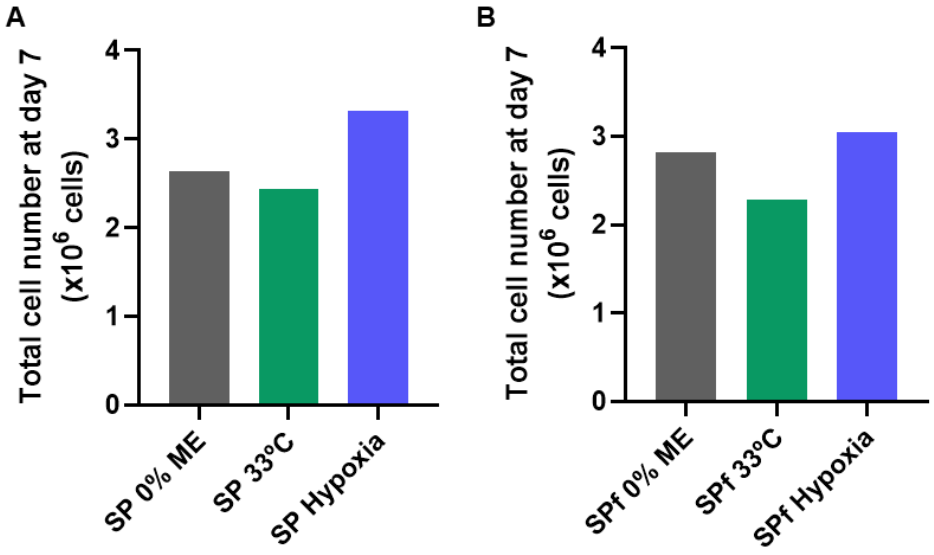

Figure 4.16- Total cell number (x10^6 cell) counted at day 7, the final of experiment, after 5 days of MSC expansion followed by 2 days of medium conditioning without any medium exchange, when using StemPro **(A)** and filtered StemPro medium **(B)**, for the conditions of normoxia at 37ºC (grey), 33ºC during medium conditioning (green), and hypoxia (5% O_2) during MSC expansion and medium conditioning (blue). SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

The size of EVs did not present significant differences between the different conditions tested, although the particle size seemed to decrease throughout time in culture, more precisely from day 3 onwards, as shown by the values of mode and mean size (**Figure 4.17**). This may indicate that the particles contained in the culture medium are larger than those produced by the cells (also seemed in time point experiment, **Figure 4.7B)** and over time of culture, there is a change in the type of particles, more precisely from particles derived from the culture medium to EVs derived from MSC.

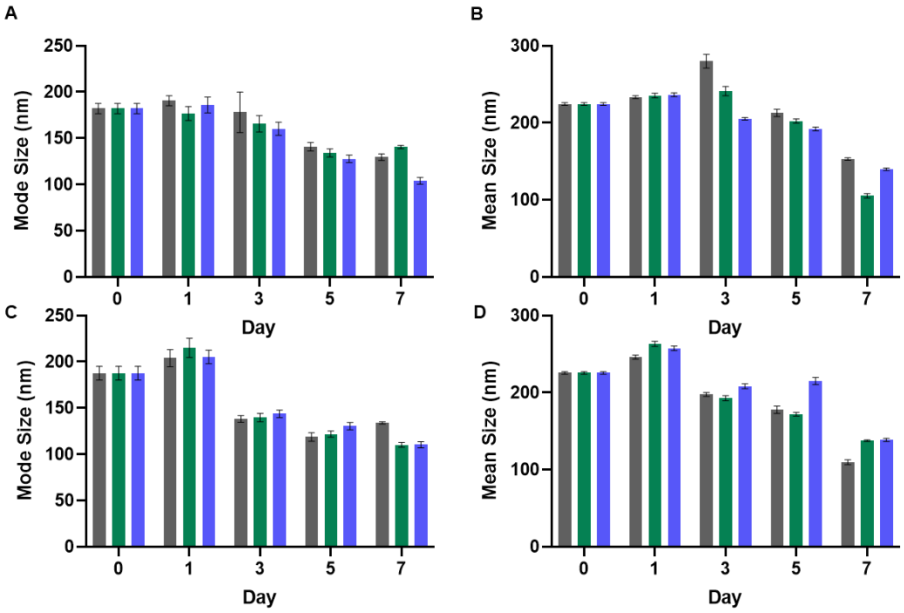

Figure 4.17- Mode **(A and C)** and mean **(B and D)** sizes (nm) from EV samples harvested at days 1,3, 5 and 7 of BM MSC culture in static systems, when using StemPro **(A and B)** and filtered StemPro medium **(C and D)**, for the conditions of normoxia at 37ºC (grey), 33ºC during medium conditioning (green), and hypoxia (5% O_2) during MSC expansion and medium conditioning (blue). Results are presented as mean ± SD.

The EV samples processed in this final experiment seemed to have similar PPR values relatively to samples from the previous experiments, in general lower than 10 and higher than 2, showing a low purity degree (**Figure 4.18**).

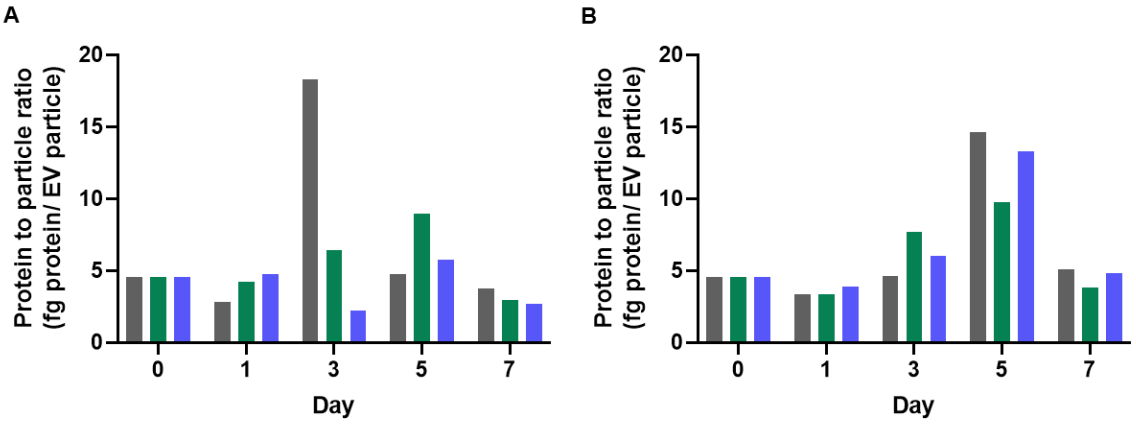

Figure 4.18- Purity assessment of EV samples harvested at days 1,3, 5 and 7 of BM MSC culture in static systems through protein to particle ratios (PPR) (fg protein/EV particle), when using StemPro **(A)** and filtered StemPro medium **(B)**. Normoxia at 37ºC (grey), 33ºC during medium conditioning (green), and hypoxia (5% O_2) during MSC expansion and medium conditioning (blue).

5. Conclusions and Future Work

In the last years, MSC therapeutic effects have been mostly associated to paracrine activity of MSC secretome including EVs, cytokines and other soluble factors. Indeed, MSC-EV therapeutic potential has already been reported and seem to recapitulate the beneficial therapeutic effects from their cells of origin, creating expectations to a promising, safe, and effective cell-free-based therapy. However, consistency, uniformity, and reproducibility in the quantity and quality of a product are necessary to produce a given therapeutic agent and it is expected that a considerable amount of EVs is needed to be applied in therapy.

In this work, different MSC culture parameters were studied under static conditions to understand their influence on MSC-EV production and investigate the best condition based on the quality and productivity of the secreted EVs. MSC-EV production comprised a stage of MSC expansion followed by a stage of medium conditioning. EVs were isolated from the conditioned media by precipitation method using a Total Exosome Isolation reagent based on PEG solution. MicroBCA was used to characterized EV samples in terms of protein concentration, NTA to determine particle number and size, Western blotting and FTIR to analyze the composition of samples, TEM to observe the morphology of EVs and PPR to evaluate purity of samples.

Firstly, cell source, donor variability, time of EV collection and medium change were evaluated in the experiment entitled “time point experiment”. Culture medium was fully renewed at day 3, and three different percentages of medium change (0%, 25% and 100%) were performed at day 6. The presence of EVs, derived from three human sources (BM, AT and UCM), in conditioned media at day 8 was identified by TEM and Western blotting. UCM MSC reached a higher cell number in the end of the experiment, comparing to BM and AT MSCs, that reached similar cell numbers. This difference could be explained by the immature nature of UCM and smaller cell size, when compared to the adult BM and AT sources. The final cell number correlated with the percentage of medium changed at day 6, as well as the concentration of EVs in the conditioned medium at day 8 (except for BM MSC). However, the higher cell number and EV concentration did not reflect in a superior EV productivity (EV/cell), which was similar between sources and percentages of medium change. BM MSC appeared to reach a greater EV productivity and disproportionate to the percentage of medium change, but it could be due to BM donor variability. Furthermore, according to FTIR analysis, 100% medium change at day 6 had the greatest influence on the molecular compositions of conditioned medium and cells at day 8, that can be related with a more significant presence of vesicles added from fresh culture medium. Overall, the MSC source and the percentage of culture medium exchange before medium conditioning seemed to not imply significant variability in EV production and productivity. Alongside, the process and the different percentages of medium exchanges did not compromise BM, AT and UCM identity since they maintained the typical MSC immunophenotype and multilineage differentiation potential.

The conditioned medium samples retrieved at days 3, 6 and 8, allowed to elucidate the evolution of uptake/production of EVs throughout MSC culture. The EVs uptake by MSC seemed to be higher in the beginning of expansion, probably to their exponential growth, and balanced with the production towards the end of the expansion and during medium conditioning, when there were more

cells to produce EVs. The analysis of these samples of conditioned media by FTIR showed differences in their molecular composition throughout culture, which could be related with that evolution in uptake/production of EVs. Although for BM and AT MSC the concentration of EVs in the conditioned media appeared higher at day 6, it was not statistically significant due to the donor variability, and therefore, the day 8 seems the best time point for EV collection. After the isolation of EVs from the conditioned media using PEG solution, it was observed that a large part of contaminants was removed, but the values of PPR were still high, indicating that the EV samples obtained were not pure. This agrees with the presence of structures that were not EVs identified by TEM in EV samples. Thus, for therapeutic applications, a second method for EV purification should be evaluated. The size distribution profile of MSC-EVs seemed regular among tissue sources and different from the culture medium sample. However, it was observed that the samples from day 3 showed a size distribution distinguished from day 6 and 8, presenting particles with higher sizes as seen in the culture medium sample. This could indicate that at day 3 the MSC culture present higher concentration of particles added from culture medium, being the concentration of MSC-EVs lower at that time, and that this concentration decreased throughout the experiment, alongside with an increase in MSC-EVs. Nevertheless, it is not guaranteed that the culture medium particles are still not present in the end of the culture.

Therefore, since the particles present in the culture medium could have contaminated the MSC-EV samples and interfered in the quantification and characterization of EVs produced by MSCs, a filtration of the culture medium was performed to try to remove those particles. However, that filtration (with 0.1 µm filter) seemed inefficient. A considerable number of vesicles were still quantified in the filtered culture medium. A filtration of the medium with a filter with lower size pores or alternative methods (ultracentrifugation) should be considered in the future for a more efficient particle removal and to remove particles below 0.1 µm. In the experiment of filtered *vs* unfiltered medium, the conditions of 0% and 100 % of medium exchange were studied again and it supported the idea that there is not much difference among themselves regarding EV productivity and 0% of medium exchange could be a better option, since it implies lower spent of medium. Here, the EV uptake/production profile seemed identical to the observed in the time point experiment, the uptake of EVs seemed higher in relation to production during MSC expansion and balanced each other during the medium conditioning period.

To finish, using the filtered *vs* unfiltered culture medium and without culture medium exchanges throughout experiment, the condition of hypoxia (5% O_2) was compared to normoxia during all the process of MSC-EVs production and a temperature of 33ºC compared with 37 ºC only during the two last days of culture after reaching 80% cell confluency. Hypoxia seemed to have impact on the EV uptake/production profile during MSC expansion and medium conditioning. However, no significant positive impact was observed in relation to EV productivity. The conditioning phase at 33ºC seemed to increase EV productivity, but more studies using other cell tissue sources and donors are necessary to confirm if the increase observed is statistically significant. Interestingly, the mode and mean sizes of EVs in this last experiment decreased throughout MSC culture. This could indicate that in the beginning of the culture there was a higher contribution of the culture medium particles (with higher sizes), and that without medium exchanges, the majority of those particles were possibly internalized by cells throughout culture and in the end of experiment remained mostly EVs produced by MSC, with lower

sizes. However, once again, it is not possible to be sure about the absence of culture medium particles in the final EV samples.

Besides the attempt of removal of the culture medium particles, and the necessary additional testing to evaluate the influence of hypoxia and 33ºC in MSC-EV production, future work can include the study of other culture parameters and the translation of the optimal culture conditions to dynamic systems, such as spinner flasks and fully controlled bioreactors. These systems present ready scalability, a more homogeneous environment, and the ability to monitor and control crucial culture parameters, providing consistency, homogeneity, robustness and standardization and thus, increasing the safety of the EV product. Different serum/xeno-free culture media and microcarriers could be tested, as well as different oxygen concentrations, temperatures, and shear stress that in this case could be better controlled. Expose MSC to inflammatory conditions, by adding pro-inflammatory cytokines to the cell culture, could also be tested since there are evidences that it might enhance the therapeutic efficacy of MSC-EVs (Park et al. 2019). Furthermore, functional studies will be required to evaluate the applicability and potency of MSC-EVs produced and to investigate their therapeutic potential, that can differ depending on the MSC source. These could include, for example, scratch assays or tube formation assays using endothelial cells to study the ability of MSC-EVs to support wound healing and promote angiogenesis (Liang et al. 2007; Vrijsen et al. 2016).

In conclusion, the present work provides insights regarding the influence of different cell culture parameters in MSC-EV production, including cell source, donor variability, time of EV collection, medium exchange, culture medium, oxygen tension and temperature, as well as about EVs degradation and adhesion to plastic throughout cell culture and the MSC-EV uptake/production profile, that can contribute to reach in the future the best quality/potency and productivity of EV products.

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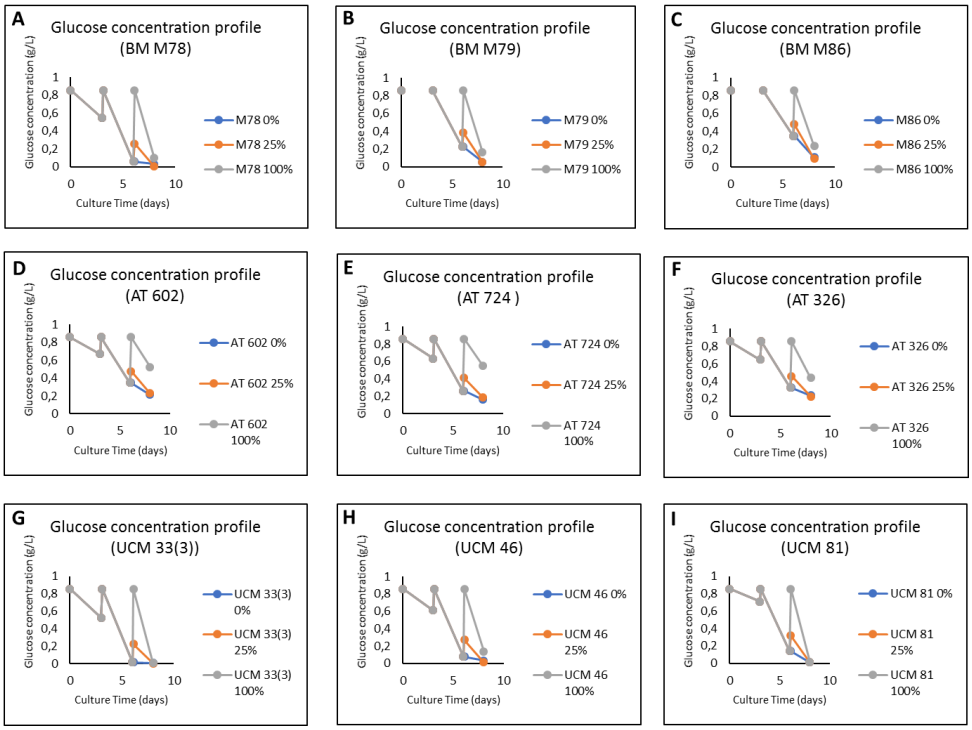
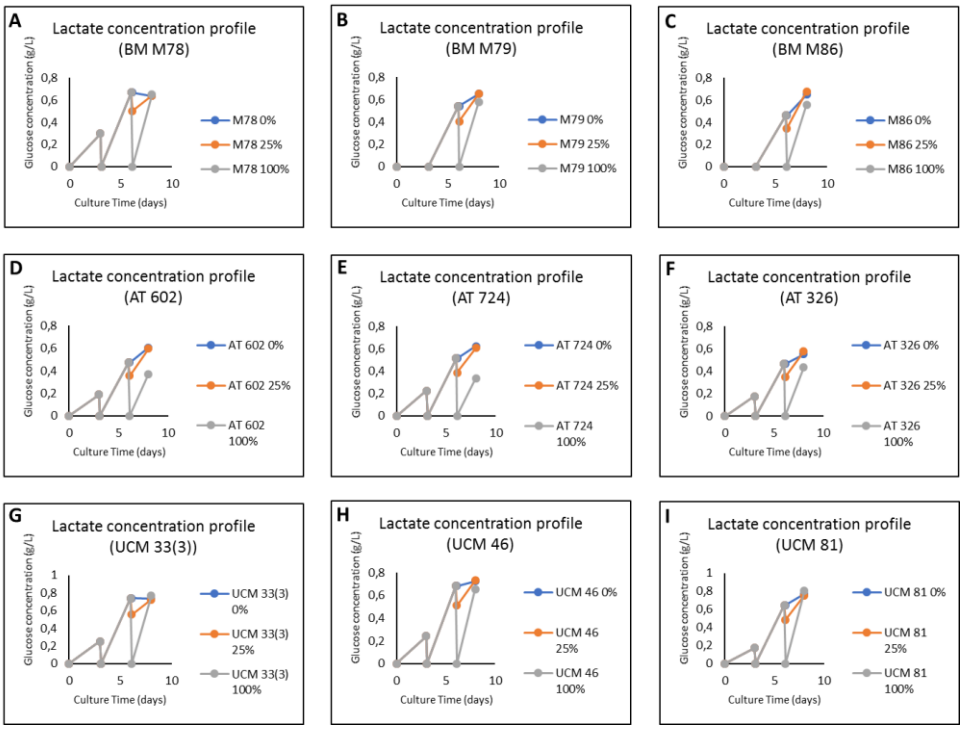
7. Supplementary Material



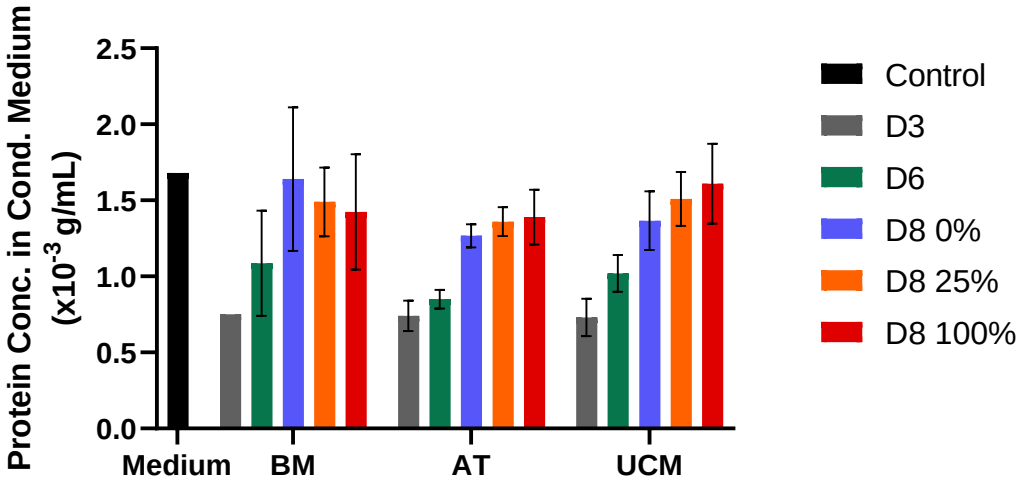
Supplementary Material 7.1- Glucose concentration (in g/L) along culture time (days), for the time point experiment, for 3 donors per cell source BM (**A** – M78A15, **B** – M79A15, **C** – M86A15), AT (**D** – L090602, **E** – L090724, **F** – L140326) and UCM (**G** – 33(3), **H** – 46, **I** – 81) MSCs in static culture. (M79A15 and M86A15 without data from day 3)



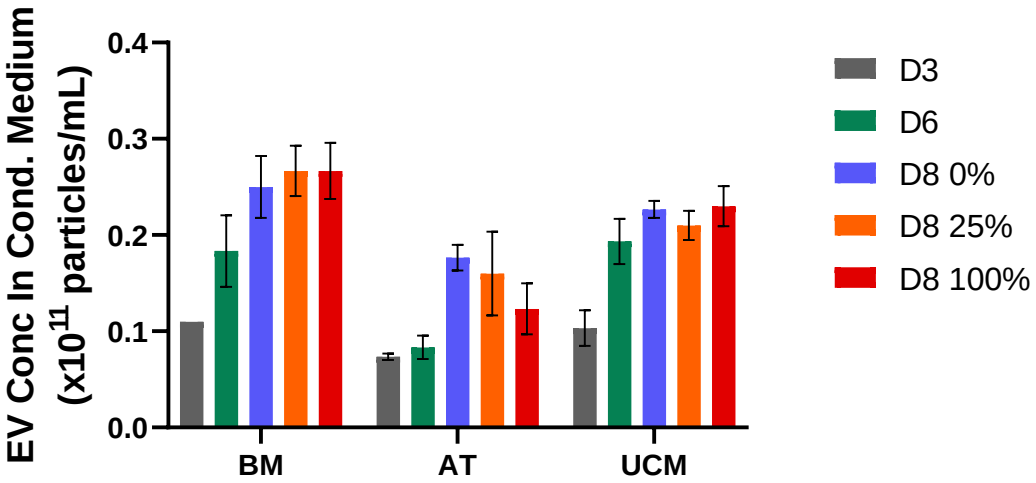
Supplementary Material 7.2- Lactate concentration (g/L) along culture time (days), for the time point experiment, for 3 donors per cell source BM (**A** – M78A15, **B** – M79A15, **C** – M86A15), AT (**D** – L090602, **E** – L090724, **F** – L140326) and UCM (**G** – 33(3), **H** – 46, **I** – 81). MSCs in static culture. M79A15 and M86A15 without data from day 3)

	BM M78 0%	BM M78 25%	BM M78 100%	AT 326 25%	AT 326 100%	UCM 81 0%	UCM 81 25%
Unstained	0.15	0.16	0.14	0.039	0.17	0.013	0.014
CD14	0.24	0.2	0.2	0.2	0.29	0.18	0.11
CD19	0.17	0.23	0.23	0.64	0.61	0.63	0.79
CD31				0.41	0.48	0.31	0.13
CD34	0.15	0.18	0.088	14.8	13.3	0.079	0.089
CD45	0.23	0.13	0.074	0.17	0.049	0.011	0.029
CD73	99.8	99.6	99.8	99.9	99.8	99.9	99.9
CD80	1.65	1.88	1.39	2.61	2.55	2.58	2.39
CD90	99.6	99.8	99.8	99.7	99.9	99.9	100
CD105	70.8	90.7	85.4	99.8	99.7	99.8	99.7
HLA-DR	1.57	1.96	1.26	0.35	0.38	0.21	0.08

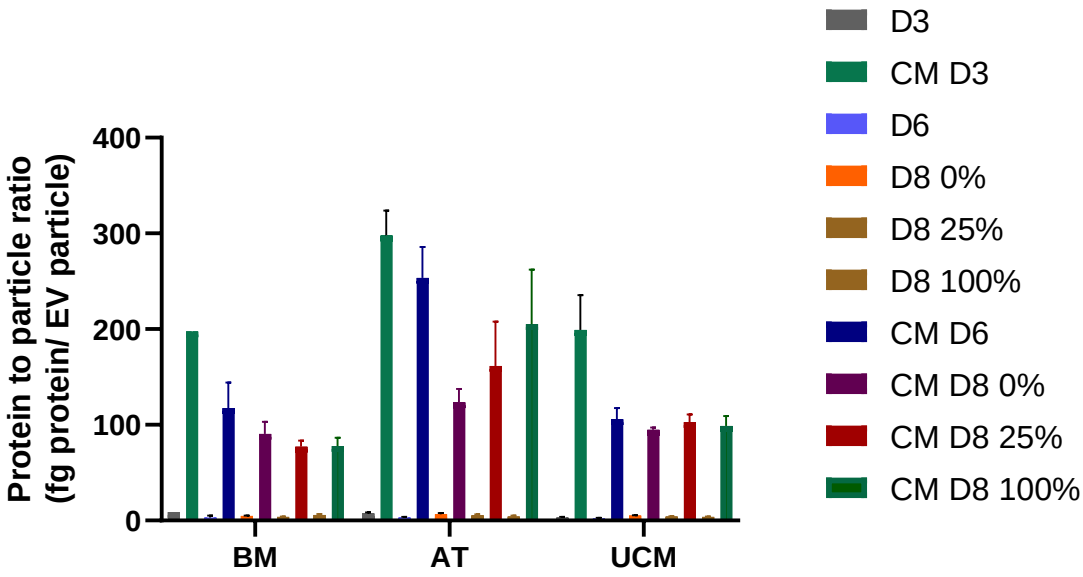
Supplementary Material 7.3- Values of marker expression (% of positive cell population) obtained through flow cytometry upon immunophenotypic characterization of BM, AT and UCM MSC after expansion and medium conditioning for MSC-EV production (total of 8 days) in static culture.



Supplementary Material 7.4- Protein concentration (x10^{-3} g/mL) in the cell culture CM from BM, AT, and UCM MSC cultures in static system, at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange). A fresh sample of the medium used as control (black). MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1).



Supplementary Material 7.5- EV concentration (x10^{11} particles/mL) in the unpurified samples of cell culture CM (CM) from BM, AT, and UCM MSC cultures in static system. Samples of CM were harvested at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange). A fresh sample of the medium used as control (black). MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). Results are presented as mean ± SEM (n = 3; except BM sample from day 3, n=1).



Supplementary Material 7.6- Purity assessment through the protein to particle ratios (PPR) (fg protein/ EV particle) of EV samples and unpurified CM samples obtained throughout BM, AT and UCM MSC culture in static system, at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium exchange). MSC from three different donors were used for each tissue source. Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1).