# UNIVERSITÀ DEGLI STUDI DI TORINO

## Research Doctorate in Veterinary Sciences for Animal Health and Food Safety
XXXII cycle

The use of mesenchymal stem cells as a tool in tissue regeneration in the equine model species

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# Summary

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# Abbreviations

**AA**: non-essential amino acids

**ACAN**: aggrecan

**ADAMTS**: a disintegrin-like and metalloprotease with thrombospondin

**AGO2**: Argonaute 2

**AP**: alkaline phosphatase

**BM**: bone marrow

**COL1**: collagen type 1

**COMP**: cartilage oligomeric matrix protein

**COX2**: cycloxigenase

**Cryo-E**M: cryo-electron microscopy

**CTRL**: control

**DAPI**: 4'6-diaminide-2'-phenylindole dihydrochloride

**DMEM**: Dulbecco's Modified Eagle's Medium

**DMSO**: dimethyl sulfoxide

**ECM**: extracellular matrix

**EGF**: endothelial growth factor

**EVs**: extracellular vesicles

**Exo-free FBS** : Exosome-depleted fetal bovine serum

**FBS**: fetal bovine serum

**FGF**: fibroblast growth factor

**FMO**: Fluorescence Minus One Control

**GAG**: Glycosaminoglycan

**GAPDH**: glyceraldehydes-3-phosphate dehydrogenase

**HPRT**: hypoxanthine-guanine phosphoribosyltransferase

**HSPA8**: Heat shock protein 8

**IFNγ**: interferon gamma

**IGF-1**: insulin-like growth factor-1

**IL-1** : interleukin-1

**IL-1β**: interleukine-1β

**IL-6**: interleukine-6

**ISEV**: Society for Extracellular Vesicles

**LAMP2**: lysosome-associated membrane protein 2

**LPL**: lipoprotein lipase

**MHC**: Major histocompatibility complex

**MMPs**: matrix metalloproteinases

**MSCs**: Mesenchymal stem cells

**MVs**: microvesicles

**NF**: neck fat

**PBS**: phosphate buffered saline

**PCR**: polymerase chain reaction

**PDCD6IP**: programmed cell death 6 interacting protein

**PFA**: paraformaldehyde

**PGE2**: prostaglandin E2

**PPARγ** : peroxisome proliferator-activated receptor gamma

**RNA**: ribonucleic acid

**RUNX2**: runt-related transcription factor 2

**RVC**: Royal Veterinary College

**SDFT**: superficial digital flexor tendon

**SF:** synovial fluid

**SOX9**: transcription factor Sox9

**TBS**: tris-buffered saline

**TEM**: Transmission electron microscope

**TFF**: tangential flow filter

**TGF**: transforming growth factor

**TIMP**: tissue inhibitor of metalloproteinases

**TNFα** : tumor necrosis factor alpha.

**TSG101**: tumor susceptibility gene 101

**VEGF**: vascular endothelial growth factor

**Chapter 1**

# INTRODUCTION

## 1. Horses in sports

Horses are known for their capacity to run at high speed (70km/h), endurance and agility, and to carry loads. Horse anatomical structure impacts on their running and speed. The size of their legs, the height of some structures from the ground (i.e. wrist) and lightness on the legs have been evolutionary adapted so that all of this influences positively on their motion mechanism (Hildebrand, 1987).

Gait in horses might be defined as a locomotion movement that requires a metabolic cost on the legs (Bertram and Gutmann, 2009). Depending on the activity, kinetics' energy may vary. This energy loss might be properly managed by correcting the support geometry of the animal. Geometrically speaking the balance and gravity center of horses is located closer to the forelimbs. This corresponds to the point between the forelimbs and hind limbs (Ross and Dyson, 2011). In respect to the horse center mass, forelimb carries a higher weight (57-58%) than hind limbs (42-43%) having proportionately higher vertical forces and impulses. The antebrachial and metacarpal segments support this function (Back and Clayton, 2013). Horse activity can modify the center balance of the horse. For example, in racehorses, the center balance is shifted to the cranial side because of the added weight of the rider. In this case, the probability of developing leg issues might be increased for the forelimbs. Whereas in draft horses leg issues are more common in hind limbs. In standardbred racehorses the balance is shifted to the center and weight is added on the back, affecting the hind limbs. In jumping and dressage horse problems on the fetlock region are commonly observed (Ross and Dyson, 2011).

### 1.1. Horse breeds

Good performances in certain activities have been evolving and improved through selection (Back and Clayton, 2013). The cannon bone index has been used as a reference to understand bone thickness i.e. legs conformation (Komosa et al., 2013). To select a horse, an athlete horse might derive from Thoroughbreds and Arabians to produce warm blood breeds (Back and Clayton, 2013). Thoroughbred (hot blood) horses (which are a type of selected horses in Great Britain known for their running capacity) differ in scapula length, being smaller in comparison with other breeds. Even though a larger bone would provide for the insertion of a higher muscle mass and to improve movement during running, these horses have lighter legs which influence their running capacity (Komosa et al., 2013). Spanish Arab horses have shown to have their morphology and endurance force in the posterior triangle area (Cervantes et al., 2009). Andalusian horses show a

better capacity of the carpal flexion allowing a large carpal motion of the joint (Back and Clayton, 2013). Running horse breeds have been evaluated and the Quarter mile horse seems to be faster than the Thoroughbred. Also, the Arabian horse is known for its running endurance capacity (Nielsen' et al., 2006). Racing distances are highly variable. As short as 400 m to greater than 160 km, high speeds should be maintained throughout the race. Speed is important and seen as a desirable characteristic. This is a combination of gait, length and stride frequency. Thoroughbreds maximal speed is around 13.7-19 m/s in galloping. In the case of sprint horses, the Quarter horse breed is known for its ability to sprint over a distance of a quarter-mile with rapid acceleration. In contrast, the Thoroughbreds which compete over short to middle distances are known for having a strong stride length (Back and Clayton, 2013).

When comparing gender performance, stallions are the ones that are better in jumping, and cross country running activities than mares. However, mares had fewer penalties i.e. in show jumping, so they seem to have better coordination in movements. Males are more easy to handle, but mares are good at training, their capacity would be also related to the rider and the type of competition (Hanousek et al., 2018). To train for competitions, an exercise program is recommended to avoid injuries and to stimulate and adapt muscle and bone. Usually, a session frequency of 3-6 days is recommended. In racetracks, 47% of injuries occur due to a lead change, 38% by whip use and 21% because of a diagonal movement (Back and Clayton, 2013).

In equine industry, more specifically in racing horses, lameness, respiratory and colic issues cause economical waste (Schlueter and Orth, 2004a). Lameness, which is considered as a clinical sign, occurs usually in the pastern rather than in the metacarpal or metatarsal region (Ross and Dyson, 2011). A survey indicated that during a year the greatest number of the day lost in training racing horses was due to lameness (68%) and that the rest of them were lame at the raining season (Schlueter and Orth, 2004a). It has been also estimated that more than 60% of lameness cases are related to osteoarthritis (OA) (McIlwraith et al., 2012a).

## 2. Anatomy of horse leg

Since horses are mainly served for sports activities, legs are really important for their performance. Front legs are the most affected because they support around 65% of horses' weight. Therefore an ideal and straight conformation of the legs is expected (Meadows, 2003). An incorrect leg conformation can predispose a horse to suffer from osteoarthritis (Schlueter and Orth, 2004a). Rear legs might not be completely straight, however when they have the typically "cow-hocked" conformation leg are less able to perform certain movements like turning or sliding (Meadows, 2003).
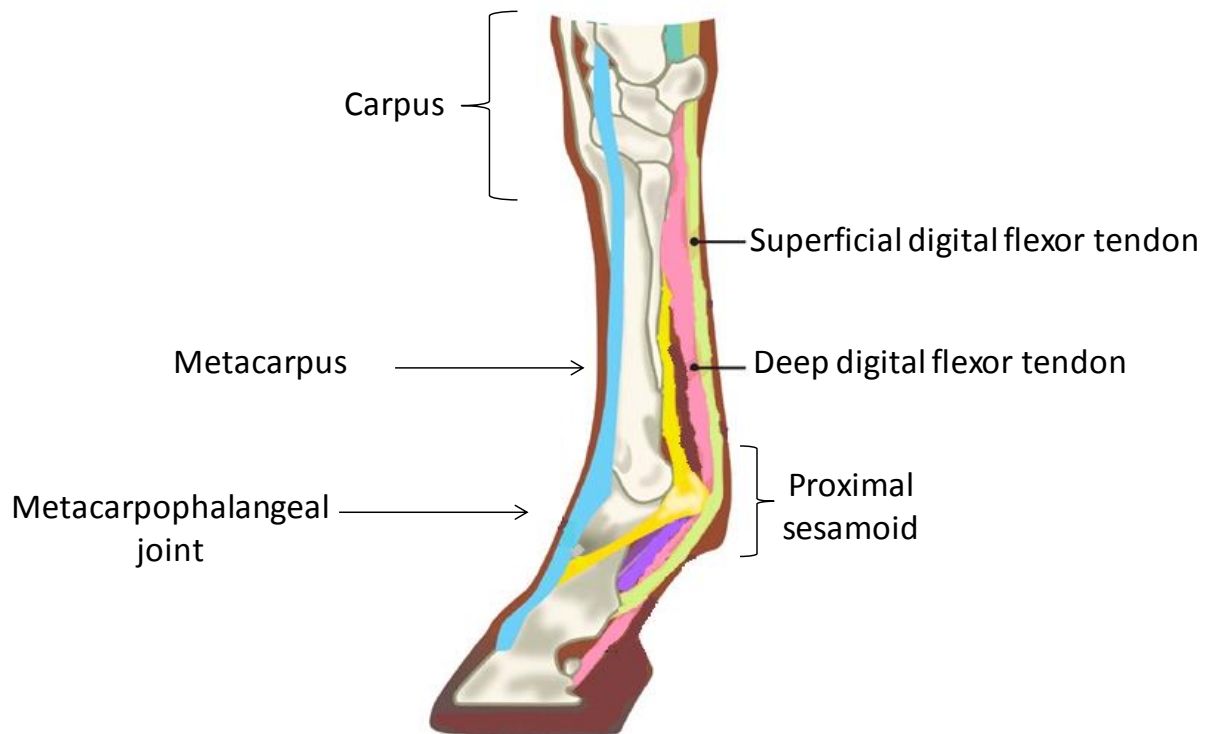
**Figure 1 Leg bone and tendons.** Main bone and tendons from the hind limb in the horse. Superficial digital flexor tendon is one of the main injured tendons in racing equines. *Image adapted from www.novobrace.com (https://www.novobrace.com/horse-anatomy-suspensory-ligament-and-flexor-tendons/)*

The metacarpophalangeal joint is known as a fetlock joint commonly suffers injuries in racing horses. Flexor tendons are mainly injured during horse activities, approximately 46% of injuries after a race affects flexor tendons. Tendons are composed mostly of water (70%), the rest of it consists of dry matter, mainly collagen and non-collagen (30%) (Dowling et al., 2000). The content of the superficial digital flexor (SDFT) seems to be richer in elastin and lubricin in the interfascicular matrix when compared with the common digital flexor tendon (Thorpe et al., 2016). The distribution of this protein in tendons remains important since it facilitates energy storage by the interfascicular sliding (Thorpe et al., 2016).

A leg comprises also joints between two bones which allows movement **(Figure 1)**. In the skeletal structure there mainly different articulation types: diarthroses which are the majority of the skeleton, the synarthroses, with reduced mobility. An articulation is surrounded by cartilage which allows protecting from shock and excessive movements. An ulcerative process in a joint marks the beginning of arthritis and osteoarthritis (Marcenac and Aublet, 1964).

A joint **(Figure 2)** is mainly constituted by ligaments that keep together the elements of a joint. They might be long or short depending on the articulation and the movement degree. Tendons are like a bandage in articulation, they allow movement and resist stretching (Marcenac and Aublet, 1964). The synovial bursa contains a liquid that allow lubrication and sliding of articulations. The value of synovial fluid also relies on the stability of joints by avoiding damage during repeated movements in locomotion. However, due to its fragility, some accidents might damage it if punctured (Marcenac and Aublet, 1964).
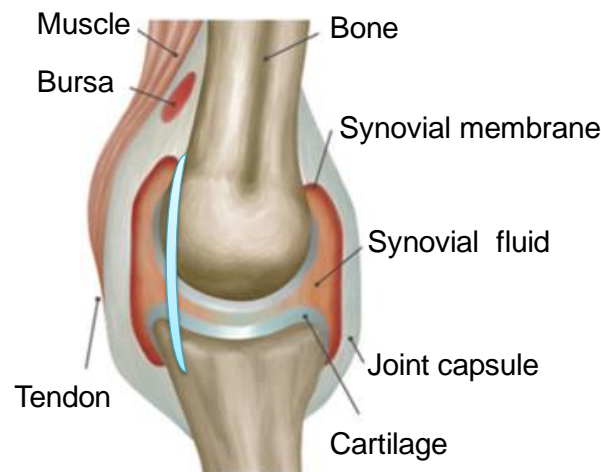


**Figure 2 Equine joint**

Equine synovial joint by the union of two bones, showing the main components that give support to an articulation (*Image adapted from "Las articulaciones del caballo" www.girovet.com*).

### 3. Osteoarthritis and tendonitis in equines

OA in horses is a degenerative joint disease. *Arthritis* means inflammation of a joint and the term osteoarthritis refers to when soft tissue is implicated (*–itis)*, contrary to osteoarthrosis (*-osis*) when such involvement is absent (Ross and Dyson, 2011). OA is a disease that commonly affects equines metacarpophalangeal joints, followed by carpal joints (McIlwraith et al., 2012a). This disease might affect the entire joint including subchondral bone, ligaments, synovial membrane, and peri-articular tissue. As mentioned before, soft tissue injuries may evolve and cause lameness in the horse (McIlwraith et al., 2012a). This mostly occurs in the legs that are constantly bearing the weight during locomotion. OA can be caused by repetitive trauma on the joint, therefore it can be considered a disease caused by everyday activity. The lack of exercise can lead to OA as well. An incorrect conformation of the legs (i.e. quarter horses that have a poor carpal conformation) and shoeing is also a risk factor for the development of OA. The age might be also a factor that can lead to OA due to degenerative modifications of the joint. However, such a relationship is not completely clear as also young horses (2 years) may develop OA (Schlueter and Orth, 2004). The pathogenesis of OA has been attributed to an initial impact shock that affects articulation and joint

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capsule acts as an attenuator of this shock. Then there are fractures or microfractures that after continuous shock damage the joint. Another cause might be that these mechanical forces alter cells and cause metabolic alteration in the chondrocytes (McIlwraith et al., 2012a). Clinical signs are swelling of the joint, pain, and deformation of the articulation. This disease can be evaluated and confirmed by radiography where the thickness of the joint can be observed (McIlwraith et al., 2012). After a shock or consecutive trauma, damage for example in certain structures like the synovial membrane is going to cause inflammation and the release of cytokines such as IL-1β (interleukine 1β) and tumor necrosis factor-alpha (TNFα). These cytokines are going to affect chondrocytes and the release of metalloproteinases is going to cause the degradation of the extracellular matrix which later is going to cause a break down of the articular cartilage (McIlwraith et al., 2012). In OA there is degeneration of articular cartilage and the development of bone. The presence of matrix degrading enzymes alters the extracellular matrix. In normal joint, chondrocytes participate in theirs metabolism to maintain equilibrium. Usually, when tissue is in a repair state, less type II collagen and proteoglycans might be expected than in a normal cartilage. Disruption of homeostasis increases catabolic processes in the joint. The presence of these catabolic functions is mediated by diverse cytokines that regulate these inflammatory processes (Ross and Dyson, 2011). As a result, proteoglycans disruption in the cartilage is observed. In the case of bone, the presence of osteophytes (bone covered by hyaline- and fibro-cartilage) is observed in the joint margins as an adaptation in OA. This might cause pain and motion decrease in the animal (Kidd et al., 2001). A deep injury can promote the proliferation of undifferentiated cells, this is, from granulated to fibrous and finally to fibro-cartilage tissue. A younger animal is going to be able to recover more easily due to active matrix synthesis and vascular supply of the epiphysis complexes in cartilage (McIlwraith et al., 2014).

Another injury that commonly affects the horse industry are issues in the supporting tendons with an incidence of 8-43% in racing horses (Dowling et al., 2000). Among the frequent tendons or ligaments injured are the SDFT, the deep digital flexor and the oblique sesamoidean ligaments (O'Sullivan, 2007; Ross and Dyson, 2011). Racing horses, jumpers and polo ponies commonly injure the SDFT, however, dressage horses usually are more affected on the deep digital flexor tendon (O'Sullivan, 2007). In racing horses, osteoarthritis and SDFT injuries are commonly due to weight and repetitive load on the legs and often occur in the forelimb. Horses are more predisposed to suffer SDFT injuries in abnormally conformed legs (Ross and Dyson, 2011). Tendon injuries take time to recover and around 20-60% of the ones that heal, 80% reinjure (Dowling et al., 2000).

Clinical signs in tendinitis are hot structures and swallow in the affected tendon. The use of ultrasonography helps in the diagnostic of this pathology. During tendinitis, an inflammatory process takes place, and there is the presence of matrix degradation enzymes, fibroblasts and inflammatory cells. The presence of cytokines (IL-1α, IL-1β, TNF-α and interferon-gamma, IFN-γ)

have been observed during SDFT pathology (Dakin et al., 2014). There might be an increment of collagen type III fibers to help to support the injured tissue. Usually, collagen type I is the most predominant in healthy horses and collagen II, III and IV are less found. A decrease of glycoprotein (cartilage oligomeric matrix proteins) concentrations are related to age but also are present during tendon degeneration (Dowling et al., 2010).

### 4. Osteoarthritis pathogenesis in joint cells

When inflammation takes place in the joint, inflammatory mediators like interleukin-1 (IL-1) are released. The role of this cytokine is to induce the release of members of the matrix metalloproteinases (MMPs) family. Other cytokines like transforming growth factor-alpha (TGF-α), beta (TGF-β) and insulin-like growth factor-1 (IGF-1) (Kidd et al., 2001) are also released. MMPs are responsible for the degradation of the articular cartilage: MMP-2 and -3 have been found with higher expression in equines during OA, with nearly a two-fold increase when compared to normal joints (Kidd et al., 2001). During OA, there are changes in chondrocytes that leads to the production of proteolytic enzymes like MMP13. Two types of chondrocytes are present in the hyaline cartilage of joints. Chondrocytes can undergo a terminal differentiation that results first in hypertrophy and subsequently in apoptosis. There is the formation of osteophytes in OA. During OA, chondrocytes produce MMP13 and type X collagen. However, this MMP13 marker is not an exclusive marker of chondrocytes hypertrophy but also of inflammation and it might be present in growing individuals (van der Kraan and van den Berg, 2012). Different stages of OA can occur simultaneously at different locations within a joint. In an initial stage when the trauma takes place, the hypertrophy-like change might be led by the transforming growth factor-alpha beta 1 (TGF-1). The presence of pro-inflammatory cytokines like IL-1β and TNF-α induce the secretion of degrading enzymes like the matrix metalloproteases 1, 9 and 13. Chondrocytes undergo proliferation, autophagy, cell death and catabolic processes (Charlier et al., 2019). Subsequently, local factors are released by the damaged cartilage and from the synovial cells, infiltrated macrophages, osteoblasts, other immune cells, and chondrocytes. Within later stages, the stimulation of fibrosis and the formation of osteophytes takes place and infiltrating inflammatory cells (macrophages) that proliferate and the presence of fibrosis and altered growth factors and the release of cytokines (van der Kraan and van den Berg, 2012).

### 5. Veterinary medicine treatments for horses with OA and tendonitis

The use of magnetic resonance and arthroscopy have served to grade the severity of osteoarthritis. Among these two, arthroscopy provides a complete evaluation of the joint. Arthroscopy also serves to evaluate intra-synovial joints and to perform surgeries that are less traumatic and painful (McIlwraith et al., 2014).

Other surgical approaches include chondroplasty which consists in smoothing the tissue that is

degraded, abrasion arthroplasty, which removes undesired bone, and microfractures to open marrow spaces and forage and drilling of the subchondral bone (McIlwraith et al., 2014; Ortved and Nixon, 2016). However, the inconvenience is that the formation of new tissue is limited and there might be bone alterations after two years (Cokelaere et al., 2016). The post-operative treatment generally consists the bandage and nonsteroidal anti-inflammatory drugs with hyaluron which might prevent the onset of lameness. (McIlwraith et al., 2014).

The use of corticosteroids in OA was the first option as initial treatment. However, the use of betamethasone esters or triamcinolone acetonide seems like a better appropriate treatment. Moreover, the use of platelet-rich plasma has been also recommended and used due to the presence of growth factors and bioactive molecules that improve the injury environment. However, even if there is the presence of a high number of platelet cells, white cells are required during the post-clinical and post-operative recovery (McIlwraith et al., 2014).

Implantation with autologous bone portions has been also used to repair tissue in horses. However, it's difficult to find available donor sites in the patient. Autologous chondrocyte implantation has been done with the aid of artificial scaffolds or in materials like fibrin, MACI® (matrix with autologous chondrocytes in a porcine collagen membrane), platelet-rich plasma vehicles, etc. However, a drawback of this technique is that sometimes the chondrocytes can dedifferentiate when cultured in monolayer. Even if there are 3D cultures where chondrocytes retain their phenotype, such cultures might need invasive implantation (Ortved and Nixon, 2016).

The tenoscopy surgery might aid in relieving tendinitis and the advantage of this technique is that is less invasive. Tenoscopy is like arthroscopy because it allows evaluating an injury by a small incision and to stimulate or eliminate undesired tissue and promote tissue regeneration. After surgery, recovery is simple and convalescence time is reduced in comparison with other procedures. Initially, the horse should remain confined in stalls for 1-2 days, then small walking periods should be considered for recovery. Lameness can be observed after this surgery and treatment with nonsteroidal anti-inflammatory drugs is recommended (McIlwraith et al., 2014).

The use of mesenchymal stem cells has been also recommended for injured tissue (Frisbie and Smith, 2010) since their use in veterinary medicine yielded promising preliminary results. Mesenchymal stem cells have been obtained and studied from rabbits, dogs, horses, cats, and cattle. In adult equines, bone marrow (BM) and adipose tissue have been largely used in comparison with another type of cells (i.e. derived from synovial membrane and synovial fluid). Cells obtained from the umbilical cord, placental and amniotic fluid have shown to improve tendinitis, however, further studies should be performed in clinics (Schnabel et al., 2013). Horses that have been treated with mesenchymal stem cells in tendinitis have shown that they could return to raising activities and after 2 years of tendinitis, they did not show reinjure. One of the main advantages of the implantation of mesenchymal stem cells in horses is that in the case of tendinitis

there is less inflammation in the tissue, there is a better alignment of tendon fibers and a more normal structure when compared with surgery (Harman, 2015). It can be also an alternative when horses are sensitive to certain drugs, to avoid toxicity.

**Table 1 Summary of the treatment options in OA and tendonitis**

| Treatment | Advantage | Disadvantage | Length of recovery* |
|---|---|---|---|
| **Debridement, micro fracture** | Considered a less invasive technique | Limitation in the repaired tissue and re-injure | 4-12 months |
| **Arthroscopy and tenoscopy** | It allows a more comprehensive evaluation of the issue. Less invasive | Not indicated in cases of limited tendinitis/OA | Up to 6 months |
| **Surgery** | Removal of the defect | Invasive | 1-24 months |
| **Implantation (cells, material, PRP)** | Biocompatible, biodegradable, easily obtained. | Might require more surgical procedures, an invasive technique | ~8 months |
| **Drugs** | Rapid relieve | Treatment of the symptoms only. | N/A |
| **Stem cells** | Less invasive, better tissue structure. Less painful, less care of the affected area. | More studies are needed to clarify cell types, immunity… | weeks-12 months |

*Time of recovery can be variable depending on the horse's age.
(Cohen et al., 2009; Schnabel et al., 2013; Smith et al., 2013; McIlwraith et al., 2014; Harman, 2015;.Ortved and Nixon, 2016)

**Chapter 2**

## Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have been suggested as a treatment option in regenerative medicine in equines. MSCs are stem cells that can differentiate *in vitro* in a different types of cells (osteocyte, adipocyte, chondrocyte, tenocyte ,and muscle cells) (Poulsom et al., 2002).

In equine orthopedics, MSCs are used to treat cartilage, tendon and bone injuries. Labeled MSCs injected in the cartilage have shown cell proliferation and an increase in the number of chondrocytes present (Poulsom et al., 2002). In the areas of injuries, apparently, MSCs engraft to the tissue, in this case (osteoarthritis) to the meniscus, fat pad and synovium (Barry and Murphy, 2004). Equines suffering from tendinitis have a difficulty to have a spontaneous recovery after an injury from local cells. Therefore, the use of MSCs seems to improve and produce extracellular matrix which resembles the tendon matrix and helps horses to recover with less probability of having a re-injure (Smith, 2008). MSCs have improved bone fractures, bone mineralization and heal bone damage (Poulsom et al., 2002).

## 1. Characteristics of MSCs

MSCs have the defining characteristics of stem cells, which are the capability of self-renewing and differentiation. In an adult individual, adult stem cells are undifferentiated cells found in tissues/organs capable of renewing themselves (that is to divide while retaining their stemness) and to differentiate into the specialized cell types that can be found in tissues or organs when needed (Stem cell, 2016). The interest in them is that they can repair and maintain tissues and organs. MSCs are multi-potent cells, meaning that they can differentiate into a variety of cell types (Pittenger et al., 1999). This characteristic has been largely studied *in vitro*, since the addition of specifically formulated media in culture results in cell commitment and terminal differentiation into osteocyte, adipocyte, chondrocyte, myocyte, etc. **(Figure 3)**. Some genes/factors that might determine cell fate are: for adipogenesis, the peroxisome proliferator-activated receptor-gamma (PPARγ), for osteogenesis, the Runt-related transcription factor 2 (RUNX2), for chondrogenesis, the transcription factor SOX9 (Sobacchi et al., 2017). Such *in vitro* differentiation is usually confirmed by staining after several days of culture (15-21 days) in a specific medium. The staining for adipose differentiation is commonly made with Oil red O to identify lipid droplets, while for osteogenic differentiation von Kossa/Safranin red or alizarin are used to stain calcium deposits. Chondrogenic differentiation is confirmed with alcian blue/ safranin O that stain glycosaminoglycans in cartilage matrix (Murata et al., 2014;  Iacono et al., 2015; Burk et al., 2017). Gene expression can also be verified to evaluate if cells underwent differentiation. For adipocytes, transcription factors like peroxisome proliferator-activated receptor gamma (PPARγ), alkalin phosphatase, lipoprotein lipase (LPL) are analyzed (Jurgens et al., 2008; Johal et al., 2015;

Barrachina et al., 2016). In osteogenesis, the expression of osteocalcin and runt-related transcription factor (RUNX2), osteonectin (Lombana et al., 2015; Barrachina et al., 2016) is assessed. Chondrogenesis is verified by the expression of collagen II, aggrecan and transcription factor SOX9 (Murata et al., 2014; Lombana et al., 2015; Barrachina et al., 2016).
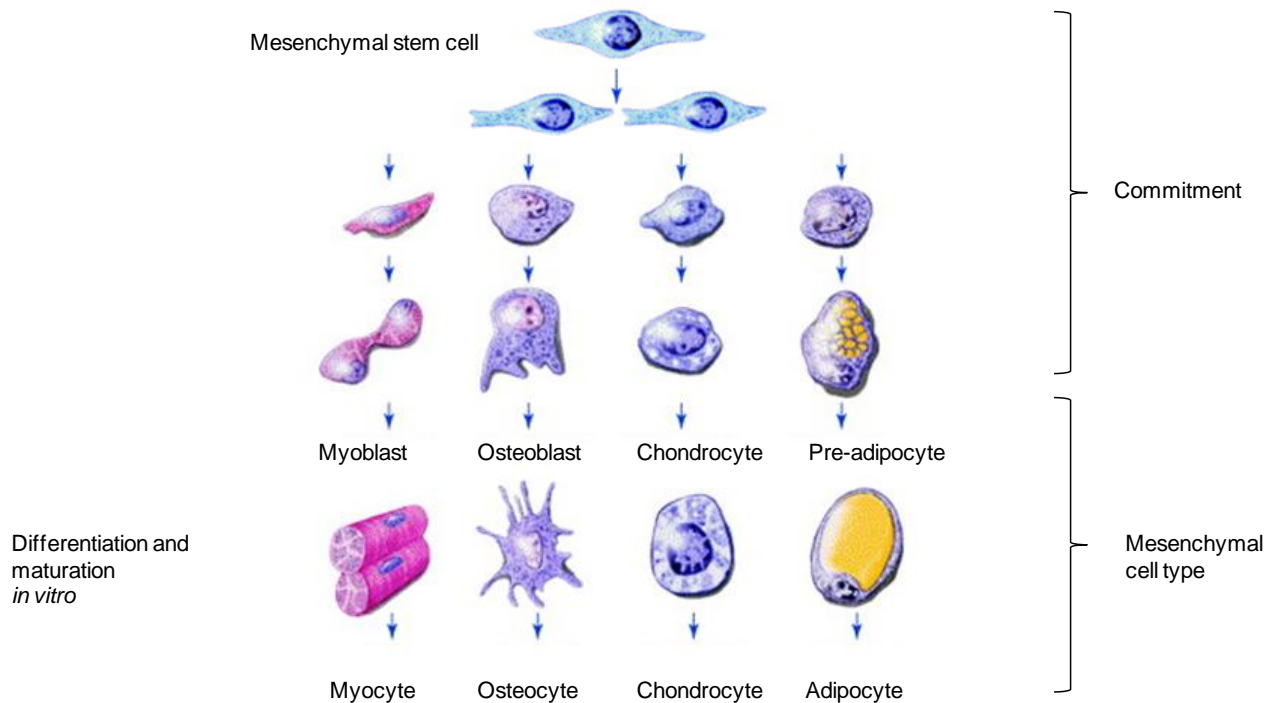


**Figure 3 MSCs differentiation types**

MSCs proliferate, go through a lineage progression to later be committed *in vitro* depending on the differentiation media added into the culture and they undergo a final differentiation towards a specific type of cell (Adapted from Risbud and Sittinger, 2002).

General defined typical characteristic of MSCs that have been suggested are: their capacity to proliferate/grow in monolayer cultures *in vitro*, expression of certain markers (CD105, CD90 ,and CD73) and their ability to differentiate into other cell types (adipocytes, chondrocytes ,and ostheocytes) (Dominici et al., 2006).

Another characteristic of MSCs is their regenerative potential which can be attributed to their capacity to release factors and molecules like the vascular endothelial growth factor (VEGF), TGF, fibroblast growth factor (FGF) among others (Schäfer et al., 2016). The release of these factors has been observed by the addition of endothelial growth factor (EGF) in *in vitro* treatment. They also improved cell expansion and wound healing.

## 2. Isolation, culture, and implantation of MSCs

The isolation of MSCs from primary tissue might vary according to the source. Usually, in the case of solid tissues, samples are aseptically collected and rinsed with phosphate-buffered saline (PBS)

to eliminate debris and residual blood content. Then, little fragments of tissue are obtained by manual mechanical disaggregation. Mechanical disaggregation is more recommended for soft tissues like adipose tissue. However, the amount of tissue required is higher, to obtain a sufficient amount of cells (Freshney, 2005). Enzymatic digestion under agitation is going to help to isolate the cells and yields a more representative number of cells from the whole tissue (Freshney, 2005). The choice of the enzymes to be used might affect the yield when different tissues are considered. Collagenase type I is commonly used for fat, epithelial, liver and lung tissues. Collagenase type II for bone, muscle, heart, cartilage and endothelial cells. However, tissue can be also digested with trypsin, dispase, etc. (Bourin et al., 2013; Worthington Biochemical Corporation, 2019). BM cells obtained from different horses can differ in their expression of marker major histocompatibility complex (MHC) class II in passage number 2. However, most of the isolated BM cells from horses are positive for MHC class II until passage number 4 *in vitro* (Schnabel et al., 2014).

The total amount of implanted cells, in the case of the typically BM-derived cells, is in the order of 4.2x10^3 cells (Ribitsch et al., 2010) to 1x10^7 cells (Smith, 2008). The number of implanted cells is not related to a better or worse clinical outcome and it might be dependent on the type of MSCs used for implantation and the type of treatment (Schnabel et al., 2013). MSCs are re-suspended in citrate supernatant obtained at the time of isolation (in the case of BM MSCs) and under local anesthesia and standing sedation they are injected back with the aid of ultrasound as a guide to find the area of interest **(Figure 4)** (Smith, 2008). After implantation, a recovery walking exercise routine program is usually prescribed in parallel with ultrasound examinations. Racing horses suffering tendinopathy of approximately 10 years that are treated with MSCs have not shown a reinjure in the following 3 years (Godwin et al., 2012). Horses treated with MSCs have shown a low significant reinjure percentage (26%) rate when compared with other types of treatments (Godwin et al., 2012). MSCs from euthanized horses might be a reliable source since they can be isolated up to 72-hour post-mortem while retaining their stem properties (Shikh Alsook et al., 2015).

In adult and young equines, there are different sources of MSCs like bone marrow, umbilical cordon, and peripheral blood but also from solid tissues like adipose tissue, synovial membrane, periodontal ligament, and skin. Other less used sources are muscle, brain, periosteum and synovial fluid, the latter being more studied lately as a promising candidate source (Ribitsch et al., 2010; Zayed et al., 2018).
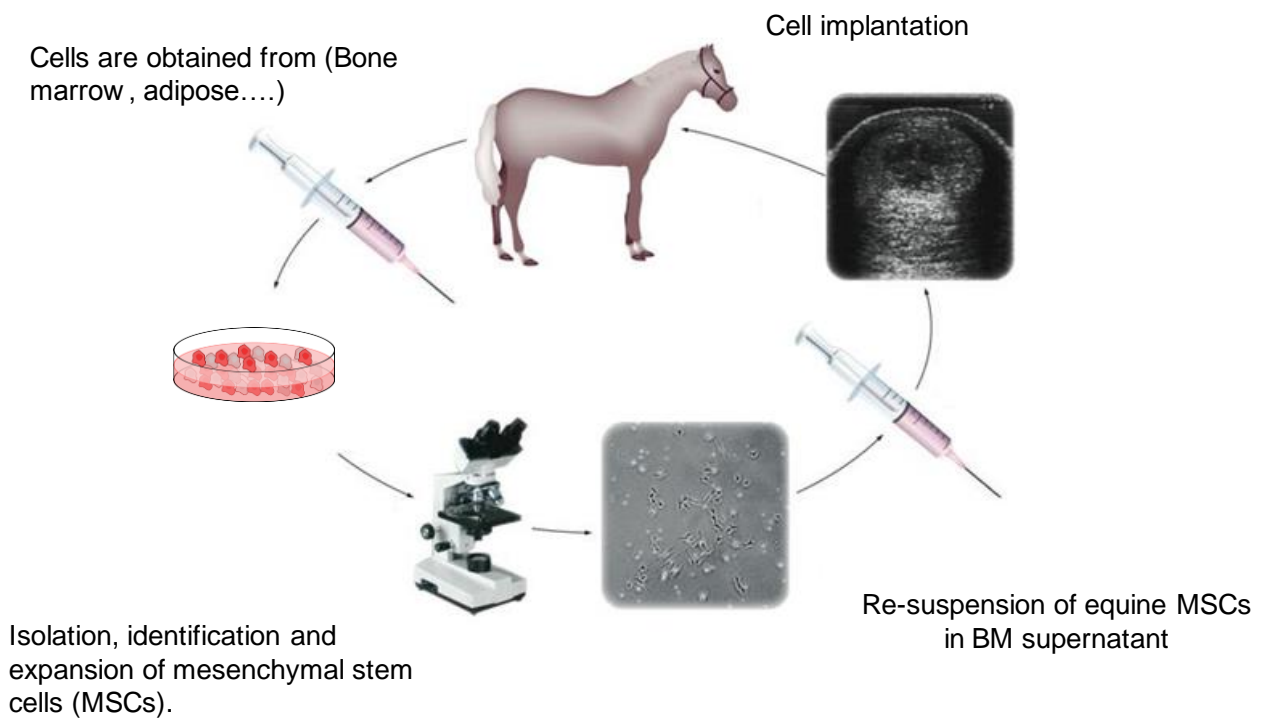
**Figure 4 Isolation and implantation of MSCs**

MSCs are isolated, cultured and analyzed *in vitro.* They are expanded to obtain a sufficient amount of cells that later are re-implanted in the damaged area by the aid of ultrasound (Adapted from Smith, 2008).

### 3. Different sources of MSCs

#### 3.1. Bone marrow

In horses, bone marrow has been one of the most frequently used MSCs sources (Smith, 2008; Schnabel et al., 2013). BM cells are mostly obtained from the sternum (among the 3-5th spaces) under local anesthesia with a syringe **(Figure 5)**. Collection can be also performed in tuber coxae **(Figure 5)**, however, it seems that a smaller amount of sample is obtained, resulting in less amount of MSCs ($6 \times 10^4$ cells/mL) when compared with the sternum ($1.13\text{-}2.19 \times 10^6$ cells/mL)(Delling et al., 2012). No significant differences are observed in the generation time (5-6 days) and growth from the cells harvested from these two sources. However, for therapeutic purposes, the tuber coxae source in middle-aged horses might not be recommended due to the high amount of cells needed for implantation (Delling et al., 2012). In horses aged 2-5 years, the sampling site should be based on sampling preference since cell growth from these two regions are similar (Adams et al., 2013). Performing two different aspirations from the same area is not very efficient since the second aspiration results in a lower cell count. Differences in cell morphology have been also observed. It seems that cells obtained from the tuber coxae are more organized than the ones from the sternum (Adams et al., 2013).

In any case, bone marrow isolation is risky both for the operators that collect the sample because they can be kicked and for the horse itself because it can fall during sedation (Ribitsch et al., 2010). In a clinical setting, MSCs have been mainly isolated from the bone marrow because of the higher number of stem cells (Smith, 2008).
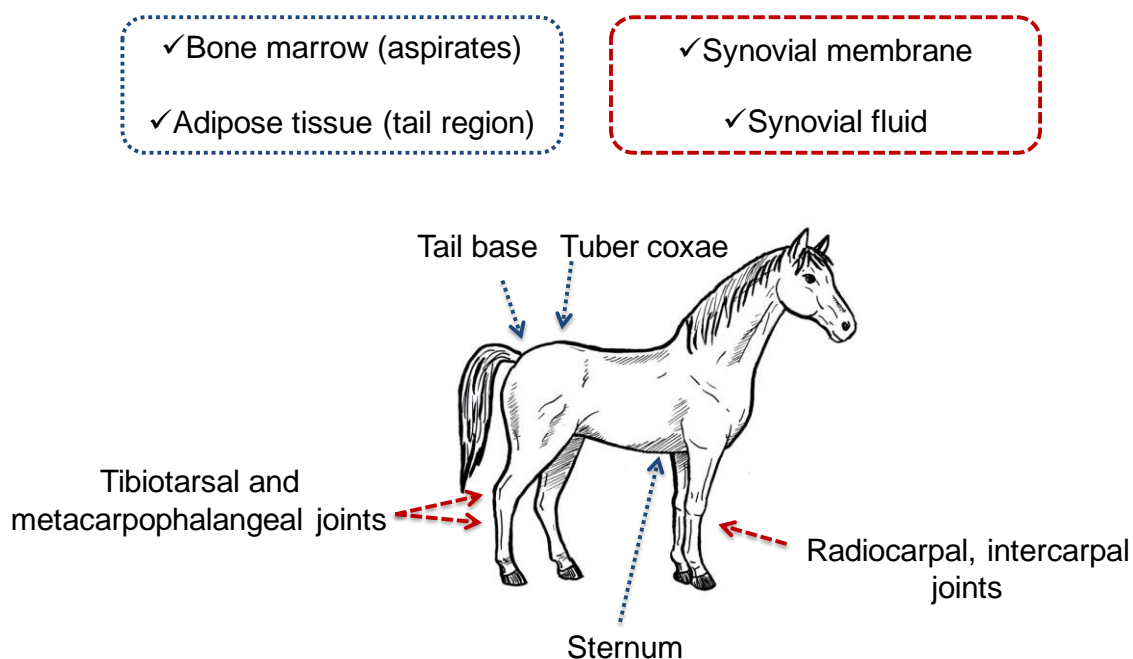


**Figure 5 MSCs harvesting regions in adult equines**

BM cells can be aspirated from live equines in a standing position as well as adipose cells from tail fat and synovial fluid under sedation and local anesthesia. Here are the main locations of harvesting.

After isolation, the cells are expanded (2-3 weeks) and implanted in the tissue of interest. BM cells are heterogeneous and they contain also hematopoietic cells but during the in vitro culture, this heterogeneity decreases with culture passages and only the fibroblast-like spindle-shaped cells remain (Ribitsch et al., 2010).

Implantation of BM cells in tendinitis lesions have shown an improvement of the morphological fiber orientation and cartilage oligomeric matrix protein (COMP). This is a protein that is in the articular cartilage extracellular matrix, it has been related to the attachment of chondrocytes to the matrix (Murray et al., 2001). COMP has been related to being present in higher or less present in cartilage depending on horse activity (Murray et al., 2001). COMP has been related to the normal physiologic orientation of the type I collagen fibers, while in non-treated with BM MSCs, tissue architecture seems disrupted or modified (Crovace et al., 2010). Horses that were treated with BM injection in the site of injury after surgery were more able to return to previous work after 24 months than those that did not receive BM implantation (Ferris et al., 2014). To reach such a conclusion, they were evaluated on lameness score damage. Using allogeneic BM MSCs from a

donor showed low adverse reaction due to the administration of anti-inflammatory drugs since the synovial structures usually have a higher reaction (meaning heat and pain in the injection area) when compared to tendons and ligaments. This adverse reaction seems to be "controlled" by the use of anti-inflammatory drugs. BM MSCs from another donor could be useful to treat horses that require faster treatment to improve their recovery time (Ursini et al., 2018). After BM MSCs implantation, some cells migrate to the lungs and liver as shown by intraperitoneal, intra-arterial and intravenous administration in mouse (Gao et al., 2001). This might cause respiratory issues depending on the number of cells that arrive at the lungs. Some of the injected cells seem to have the ability to "home" to the site of injury, which is to migrate and implant where the tissue has been damaged  (Gao et al., 2001). BM cells labeled with technetium-99m for medical diagnostics showed that in certain conditions, the cells that were injected intravenously were not able to arrive to the injury site, while the cells that were administrated directly in the lesion had more successful engraftment where approximately 24% of the cells were retained after 24 hours. However, it seems that cells have a low migration capacity reaching the site of injury (Becerra et al., 2013). When repeated intravenous injections of allogeneic BM MSCs were performed in equines, there was just an increase of CD8T+ cells but not of other immunologic response. Such behaviour suggests that the administration of allogeneic cells in healthy equines  could be considered safe (Kol et al., 2012).

Bone marrow-derived MSCs are positive to CD29, CD44, CD90, CD105, CD11α/18 and have a low or negative expression of CD14, CD34, CD45, CD79α, CD73 and MHC II (Paebst et al., 2014; Iacono et al., 2015).

### 3.2. Adipose tissue

Similar to bone marrow, adipose tissue is a source frequently used in equine regeneration therapy. It is largely available and easy to obtain (Ribitsch et al., 2010). It is usually harvested from the region above the dorsal gluteal muscle and the base of the tail **(Figure 5).** This area lacks large veins that could make the sampling difficult (de Mattos Carvalho et al., 2009). An incision is made by the aid of L-block infiltration anesthesia, which means horse is sedated with xylazine, for example, and local anaesthesia is infiltered subcutaneously at different sites forming a letter "L" (de Mattos Carvalho et al., 2009). Adipose tissue can be collected either with a lipoaspirate with a cannula or by tissue excision (Metcalf et al., 2016). Adipose cells show a fibroblastoid shape when cultured on plastic. It has been shown that the frequency of adipose cells is higher and that there can be a total cell yield of $4.5 \times 10^5$ cells per gram of fat (Ribitsch et al., 2010). It has been reported in equines that from approximately 10-20 grams of adipose tissue 16.3 million nucleated cells can be obtained (Frisbie et al., 2009). Mesenteric cells have shown to have the highest nucleated cells per gram and cell viability in equines, followed by tail and inguinal fat deposits (Metcalf et al., 2016). Adipose-derived equine cells have shown a linear increase during 8

consecutive passages *in vitro,* while BM-derived cells show this pattern only until the 5th passage (Iacono et al., 2015).

When comparing the administration of BM MSCs against adipose MSCs in OA cases in equines it was observed that the concentrations of prostaglandin E2 (PGE2), related to inflammation were decreased by the adipose cell fraction administration, but it was not statistically significant and important when compared with BM MSCs. In fact, the administration of adipose MSCs caused an increment of the concentration of tumor necrosis factor-alpha (TNF-α) in the synovial fluid concentration, which is related to inflammation (Frisbie et al., 2009). However, other studies show that after implantation of adipose cells the grade of the tendon lesion was reduced and that after 12 months post-treatment the horses returned to racing competitions (Iacono et al., 2015). To identify adipose MSCs, they should be positive to CD90, CD105, CD29, CD11α/18 ,and CD44, while they should have a low or negative expression of CD34, CD45, CD73, MHC II ,and CD14 (Paebst et al., 2014; Iacono et al., 2015).

### *3.3. Synovial tissues*

The synovial membrane is related to cartilage since its origin is from a common source of progenitor cells and they synthesize high amounts of hyaluronan (Ribitsch et al., 2010). Synovial fluid was thought to be just a liquid within the joint articulations with the function of reducing friction during movement. However, it has been suggested as a source for MSCs (Prado et al., 2015). Synovial membrane and synovial fluid can be obtained during arthroscopic **(Figure 5)** surgery from equines at the tibiotarsal and metacarpophalangeal regions (Prado et al., 2015). Synovial fluid has been also isolated from radiocarpal, intercarpal and tarsocrural joints with a syringe or with microtainer tubes **(Figure 5)**. The hind limb/forelimb harvesting site choice seems to be depending on the manual skill/ease of collection by the operator that is sampling (Murata et al., 2014). Cells derived from synovial membrane and fluid present a fibroblast-like morphology and fusiform shape. Synovial derived cells are round after seeding, but after a few days (usually around 4 days) they acquire their typical morphology (Zayed et al., 2017). A period of adjustment or lag phase without proliferation has been observed after culturing them *in vitro* in both synovial membrane (24 hours), and synovial fluid (50 hours) samples (Prado et al., 2015). Synovial membrane cells seem to decrease their proliferation rate between 48 and 144 hours, while synovial fluid cells seem to maintain their proliferation (Prado et al., 2015). Both the synovial membrane and synovial fluid cells take around the same time to reach confluence after being trypsinized and passaged (Fülber et al., 2016). However, in synovial fluid and membrane-derived from horses suffering osteochondrosis, cell population doubling time seems to be significantly faster than one of healthy equines. Healthy synovial fluid and membrane-derived cells show more extracellular matrix deposition than cells with osteochondrosis and OA (Fülber et al., 2016). Synovial fluid cells show a higher deposition of glycosaminoglycan (GAG) than bone marrow cells. GAGs are involved in the development of

connective tissue and cartilage structure (Zayed et al., 2017). The transcriptor factor SOX9 in chondrocytes is related to cartilage formation and has been evaluated in synovial fluid cells and found to be highly expressed. Synovial derived cells seem to be an attractive source of MSCs (Zayed et al., 2017). However, the implantation of synovial cells has not been largely studied as bone marrow derived cells. The implantation of equine synovial fluid MSCs in murine articulations show a higher cartilage regeneration profile by the expression of collagen II than bone marrow cells (Zayed et al., 2018b). It has been reported that autologous and allogeneic synovial fluid cells implanted in equine joints caused an increase in lameness and neutrophil count (Zayed et al., 2018a). The presence of lameness after cell implantation therapy has also been reported by Smith and colleagues (Smith, 2008). However, the evaluation of cells before implantation is always recommended (Zayed et al., 2018a) and further studies on synovial fluid-derived MSCs are required.

Synovial membrane and synovial fluid MSCs are positive to CD90, CD105, CD11α/18, CD117,CD133 ,and CD44 and should have a low or negative expression of CD34, CD45,CD73, MHC II ,and CD14 (Paebst et al., 2014; Iacono et al., 2015).

### 3.4. Other tissues

In equine adults, other tissues like peripheral blood, obtained from the jugular vein have been successfully used to recover MSCs. Isolated MSCs from blood can adhere to plastic and differentiate into osteocytes, chondrocytes and adipocytes. They were positive for CD29, CD44, CD90, and CD105. They presented a negative expression of CD45, CD79α ,and MHC II (Spaas et al., 2013). After several passages (5 to 6), blood-derived cells seem to change their morphology to the spindle shape and later they lost their proliferation reaching only passage 5 or 6 (Koerner et al., 2006). Almost 50% of the cells from blood were lost after trypsinization and after thawing from liquid nitrogen, compared with the ones derived from bone marrow (5-10%). When implantation was tested, peripheral blood-derived MSCs that were supposed to be implanted declined in number during the 12 hours of transportation to the location of the horses (Broeckx et al., 2014). The *in vitro* culture and the addition of growth factors (dexamethasone, indomethacin and insulin) might improve their proliferation capacity (Giovannini et al., 2008). However, this source remains consistently less used than bone marrow, despite the ease of collection.

**Chapter 3**

## Extracellular vesicles

Among the advantages of using MSCs in regenerative therapy, it has been suggested that they might avoid immune recognition (Zhao et al., 2016). Moreover, their safe use has been proved *in vitro* showing that they maintain their functional characteristics and phenotype for a long time (2 months) without suffering cell modification/transformation (Zhao et al., 2016). However, they have been shown to participate in the recruitment of neutrophils and activation of circulating macrophages and tissue monocytes. Generally speaking and as mentioned before, implanted MSCs act by regulating immune-responses, by increasing cell proliferation within the endogenous tissue and by transferring of vesicular components (Prockop and Youn Oh, 2012). Also, they possess the capacity to induce or modulate tissue remodeling and reduce the entity of damaged tissue when an injury takes place. Overall they lead to the improvement of tissue function (Kinnaird et al., 2004). When MSCs are delivered to an injured region after implantation, they stimulate local stem and progenitor cells and they might participate in the homing of these cells to the site of injury (Kinnaird et al., 2004). MSCs that are located in damaged regions act through a paracrine mechanisms by secreting growth factors (VEGF, HGF, IGF1…), cytokines (IL6, TNF…) but they might also directly participate in extracellular matrix (ECM) remodeling by producing matrix metalloproteases such as MMP2 and MMP9 but also inhibitors of such enzymes as TIMP1 and TIMP2. Moreover, MSCs can stimulate or modify their surrounding cells' secretion (Yao et al., 2015). It has been proposed that the positive effect of MSCs during implantation might be also due to their capacity to secrete bioactive factors like the release of extracellular vesicles (EVs) or MVs (microvesicles) (Tetta et al., 2012)

## 1. Extracellular vesicles

Cells secreted vesicles have been classified and named in a variety of ways in literature, but generally, they are classified depending on their physicochemical characteristic: size, an appearance by electron microscopy, lipid composition and depending on their origin. Extracellular vesicles are heterogeneous, so this general term is replaced according to each of the different particles released by the cell and which can be: exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles and apoptotic vesicles (Thery et al., 2009). According to the International Society for Extracellular Vesicles (ISEV), the term extracellular vesicles might refer to exosomes, microvesicles, apoptotic bodies, matrix vesicles, ectosomes, prostasomes, membrane particles and shedding vesicles. EVs in general, do not share the same characteristics and moreover, there are no established markers for each specific type and size of EVs (Malda et al., 2016). Extracellular vesicles' size ranges from 30 to 1000 nm (Thery et al., 2009; Tetta et al., 2012). There are diverse EVs classifications but, generally speaking, exosomes are often referred to EVs that are derived from the internal compartments from the endosomal membrane by

exocytotic process (**Figure 6**). They are enriched in cholesterol, contain lipid rafts and expose phosphatidylserine. They are from a size range of 30-120 nm. Microvesicles are from 100-1000 nm and they derive from the plasma membrane by budding from the membrane and they expose phosphatidylserine (**Figure 6**) (Thery et al., 2009; Tetta et al., 2012).

EVs participate in cell-to-cell communication and are carriers of proteins, mRNA, miRNAs, growth factors and other signaling molecules (Lötvall et al., 2014). The importance of transferring their cargo information from one cell to another one is that they support or perturb different physiological processes like modifying their microenvironment and determining cell fate (i.e cell growth mechanisms, phenotype, differentiation ,and tissue remodeling) (Tetta et al., 2012; Nawaz et al., 2016). When cells release EVs, the neighboring cells are going to be influenced by uptake by membrane fusion or by interaction with their membrane receptors. The content or type of EVs depends on the state of the releasing cells. For example, EVs from cancer cells are going to be different from EVs from normal cells and those produced by cells exposed to pro-inflammatory cytokines (Tetta et al., 2012; Harting et al., 2018).
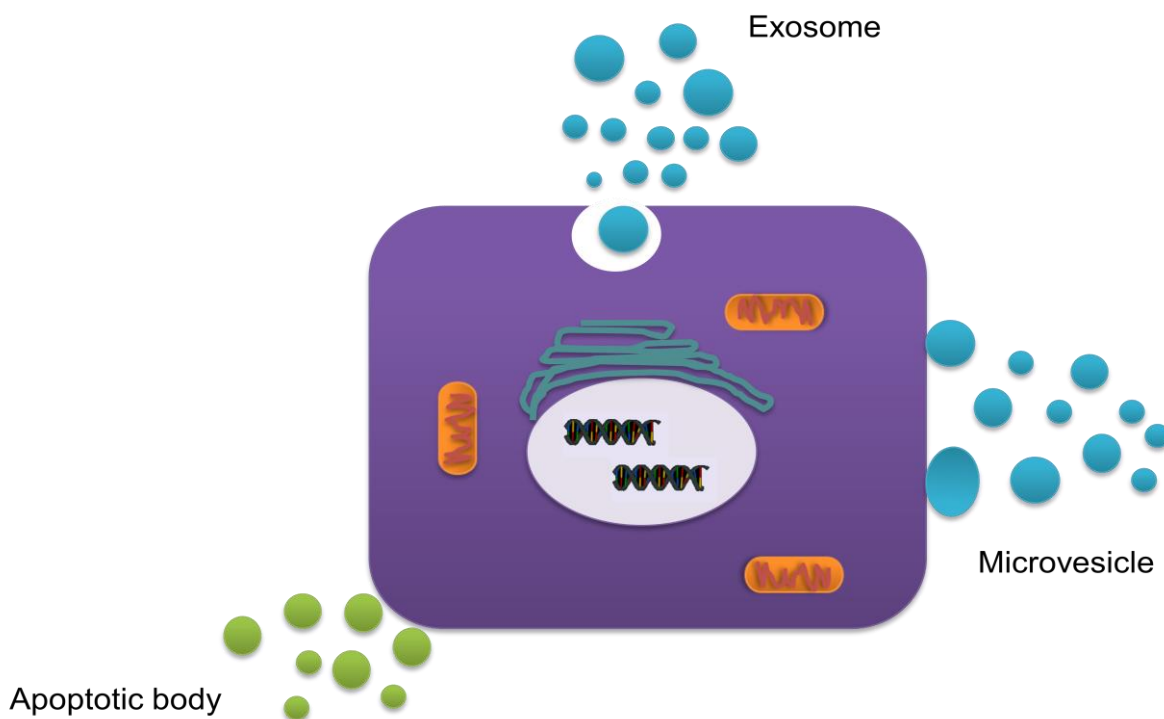


**Figura 6 Secretion of Extracellular vesicles**. The release of EVs into the extracellular space can result by budding plasma membrane (microvesicles) they might contain cytoplasmic components. While the exosomes are released by exocytosis containing intraluminal components. Apoptotic bodies can be also released by the cell with non-determined origin.

The function of EVs in the transport of certain molecules can also be diverse. Some EVs can transmit information at the cell surface and not deliver their content. During cancer, cell EVs transfer proteins and potentially toxic components that are loaded into the EVs to be disseminated to other cells (Mathieu et al., 2019).

EVs have also been implicated in the regulation of the extracellular matrix components. They might carry with them some transcription factors or other regulatory proteins that might affect the cytoskeleton allowing for morphology changes and at the same time acting as pro-survival signal and growth stimulation (Antonyak et al., 2011). It has been shown that in mammary cancer, surrounding fibroblasts are affected and that their activity might be modified to improve cancer cell development (Schwager et al., 2019). EVs-derived from these cancer cells impair fibroblast proliferation and cause an increase in traction stress on matrix stiffness. However, the effect of EVs depends on the type of cell since normal cells have no such influence on fibroblasts this behavior relates to the malignancy of cancer cells (Schwager et al., 2019).

On the other hand, EVs can travel within the blood-stream transporting as their content information from the donor tissue. For example, during cancer, EVs-derived from brain tumor cells are released into the bloodstream. The detection of specific EVs content might serve then as a biomarker for some diseases (García-Romero et al., 2017).

EVs have been recognized by their immune properties due to the presence of immune-related miRNAs (miR-106a, miR-181a, and miR-451) in colostrums-derived EVs. However, their EVs' structure should be preserved to perform their biological functions (Sun et al., 2013). For instance, if membrane vesicle is affected by i.e. ultra-sonication during an *in vitro* experiment, this reduces their effect on cells and shows the importance of the preservation of EVs structure (Sun et al., 2013). The presence of this type of immune-modulatory miRNAs is higher in colostrums than in milk (Sun et al., 2013). The presence of miRNAs related to the immunological activity in derived bone marrow EVs seems to decrease with age, therefore reducing their capacity to act against cytokines and inflammatory cells (Fafian-Labora et al., 2017). EVs have been also related to coagulation processes as they have been proved to accelerate this process (Khaspekova et al., 2016). Derived EVs from endothelial cells and monocytic cells produce Tissue factor (a protein that initiates the thrombin formation in the coagulation cascade) in the presence of bacterial Lipopolysaccharide (Khaspekova et al., 2016).

## 2. Isolation of extracellular vesicles

Analyses about genes and specific transmembrane proteins in the EVs have served to identify them. Depending on the type of cell, certain specific transmembrane proteins might be low, medium or highly expressed (Théry et al., 2006). These transmembrane proteins and other type of proteins in EVs have been identified mainly by western blot analyses (Nguyen et al., 2018). It has

been reported that EVs-derived from MSCs express tetraspanin CD63, CD9, and CD90. Other proteins have been identified and used as markers to identify EVs i.e. proteins in EVs-derived from urine, melanoma and dendritic cells such as Alix and TSG-101 (Théry et al., 2006). However, some proteins like the latter might not be detectable by western blot in MSCs derived EVs (Nguyen et al., 2018). There are another type of proteins that have been reported to be present in EVs like MHC I, MHC II, CD81, calnexin, MFG-E8 (Théry et al., 2006), however, not all EVs' cell types might express them. Cell surface markers CD9, CD90, CD63, CD81 and Lamp1 have served to isolate EVs by immune-affinity and to identify MSCs EVs by flow cytometry or by protein expression (Bandeira et al., 2018; Nguyen et al., 2018; Klymiuk et al., 2019).

In literature, several methodologies to isolate EVs have been described and they are from most to least commonly used: ultracentrifugation, density gradient separation, filtration, size exclusive chromatography and precipitation with polymers (Konoshenko et al., 2018).

Different centrifugation speeds (with one or two ultracentrifugation cycles) have been proposed as a technique to obtaining EVs (Konoshenko et al., 2018). Usually, this methodology consists of performing a low-speed centrifugation to eliminate particles in suspension followed by other two or three rounds of centrifugation to precipitate cells (either dead or alive) and cell debris. The resulting supernatant is going to be transferred to a clean tube then a high-speed ultracentrifugation (>100,000g) for a couple of hours is performed. One or two rounds of centrifugation might be recommended depending on the protocol. Later on, the EVs pellet can be recovered (Théry et al., 2006; Konoshenko et al., 2018). The speed and timing might be adjusted depending on the type of rotor and the type of ultracentrifuge (Théry et al., 2006). Even though longer centrifugation times might increase the number of recovered EVs, it is recommended that the ultracentrifugation should not exceed four hours because longer times might also increase the presence of impurities in the samples (Konoshenko et al., 2018). EVs isolated by ultracentrifugation have shown the presence of 20-250nm particles and the analysis of proteins and RNAs expression is possible after using this isolation method (Konoshenko et al., 2018). It has been reported that the highest amount of ribonucleic acid (RNA) was found after the ultracentrifugation isolation in comparison with other methodologies (i.e. commercial kits) (Van Deun et al., 2014).

Density gradient separation has been recommended for separating subcellular components (mitochondria, peroxisomes, and endosomes). This separation technique requires ultracentrifugation and the difference between the typical or differential ultra/centrifugations is that there is the use of compounds (caesium salts or sucrose solution) that are used to form a density gradient. Some of these substances i.e. a sucrose solution are loaded at the bottom of a tube and after ultra/centrifugations EVs segregate at a specific density in the gradient (Théry et al., 2006). The separation of EVs is based on their buoyancy density or sedimentation. This methodology also provides different fractions of EVs according to size and it allows to isolate more pure EVs,

proteins ,and miRNAs in comparison with ultracentrifugation or commercial kits (Konoshenko et al., 2018).

Another separation technique is size exclusion chromatography, which has been used to isolate EVs from plasma and urine. EVs can be separated by size, this is possible due to the use of different columns in the equipment. This method allows obtaining EVs that show a larger number of EVs markers to identify them (i.e. CD63, Alix, and tumor susceptibility gene 101 (TSG101), etc.) and diminishes non-EVs proteins. One of the disadvantages of this method (**Table 2**) is the low amount of EVs and the expensiveness in the costs of the material used for EVs isolation (Konoshenko et al., 2018).

The polymer-based precipitation technique allows isolating EVs in the range of 30-200 nm size. There are some commercial kits i.e. Invitrogen™ Total Exosome Isolation Reagent which consist on harvesting the media from cell culture, followed by centrifugation to remove media and an overnight incubation of the pellet with a reagent. Later, there is another centrifugation and recovery of the pellet containing the EVs. There is another type of polymer-based precipitation buffer with size-based purification (Exo-spin™, Cell guidance systems) in which there is an additional step in which the EVs suspension is loaded into spin-columns (with a 30 nm pore size of the resin) resulting in a more purified sample (Niu et al., 2017). Using this method allows to recover RNA as in other methodologies. However, protein yield seems to be 3 times higher when using commercial kit i.e. ExoQuick™ (System Biosciences) than the ultracentrifugation methodology for EVs isolation (Van Deun et al., 2014). When analyzing the expression of EVs proteins (such as the Argonaute (AGO2) protein that could be involved in the miRNA enrichment of EVs (Iavello et al., 2016) that is found within EVs and tumor susceptibility gene (TSG101, a core component of the endosomal sorting complexes required for transport), AGO2 seems only expressed when extracting EVs by the ExoQuick™. However, the protein exosome marker TSG101 seems to be absent (**Table 2**)(Van Deun et al., 2014).

Another isolation technique that has been reported is filtration. Filtration methodologies and microfiltration (using membranes with different pore sizes) could help to isolate EV in different sizes. However, their use has shown that the isolated EVs might be contaminated and that some EVs remain in pores of the filters (**Table 2**) (Momen-Heravi et al., 2013). Another filtration methodology is the tangential flow filtration, which allows isolating a good amount of media (8-10mL) and recover $1\times10^9$ EVs/mL with a homogeneous size (Harting et al., 2018). The tangential flow filtration consists of filtrating the harvesting media, to remove cells and debris. Then, the sample is loaded in a tangential flow filter (TFF) which is a device that filters samples using a determined pressure. This reduces the volume of sample eliminating waste and PBS buffer is added in the system to replace the one lost (Harting et al., 2018). The remaining volume will contain the EVs. This method selects and cleans from contaminants or proteins and selects EVs.

The inconvenience of this methodology is the requirement of specific instruments and a more complicated protocol (Konoshenko et al., 2018).

Generally speaking, it seems that filtration is used in combination with ultracentrifugation (Konoshenko et al., 2018). Isolation of EVs might depend on the type of target analysis required for each study. Commercial kits seem to result in a higher amount of EVs and are less time-consuming. However, different centrifugations and ultracentrifugation in combination with filtration would result in either higher yields or better performance in downstream applications such as protein analysis and imaging (Serrano-Pertierra et al., 2019).

**Table 2 Methodologies for the isolation of EVs**

| Method | Advantage | Disadvantage |
|---|---|---|
| Ultracentrifugation | • Less expensive<br>• Easy to perform<br>• Commonly used | • Requires equipment<br>• Risk of contamination |
| Density gradient | Obtainment of a more pure EV sample | Risk of contamination from material remained in the EVs density fraction |
| Size exclusive chromatography | Separates molecules of different sizes | • Low yield<br>• Expensive |
| Filtration | • Isolation of different EVs sizes<br>• Separates contaminants | • Low recovery of sample<br>• Deformation and disruption of EVs<br>• Requires equipment |
| Precipitation with polymers | • Easy to perform<br>• Does not require special equipment | • Residual polymers contamination<br>• Difficulty to perform further analysis in the EVs |

(Théry et al., 2006; Taylor and Shah, 2015; Konoshenko et al., 2018)

## 3. Analyses and characterization of EVs

To identify EVs different and general methodological analyses have been carried out in literature: the use of a nano tracking particle system, transmission electron microscopy (TEM) and flow cytometry to analyze the EVs, while their content has been examined by protein analysis by western blot, RNA and microRNA, (Théry et al., 2006).

*Nanoparticle tracking analysis*

This is the main technique to assess the concentration and size in a EVs sample (Ramirez et al., 2018). This technique consists of analyzing particles in suspension visualizing them in real-time (**Figure 7**). First, samples are loaded on a microscope stage. Second, a laser is used to illuminate the sample and a camera records the light scattering caused by the particles and tracks their movements in suspension (**Figure 7**). The particles in suspension have Brownian motion which is analyzed using a software that estimates the number and size of particles through a Stokes-Einstein equation: where D=diffusion coefficient of a spherical particle; T=absolute temperature; $N_A$=Avogadro's number; R= gas constant (Carr and Wright, 2010).

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$$D = \frac{\breve{R}T}{N_A} \frac{1}{6\pi\eta a}$$
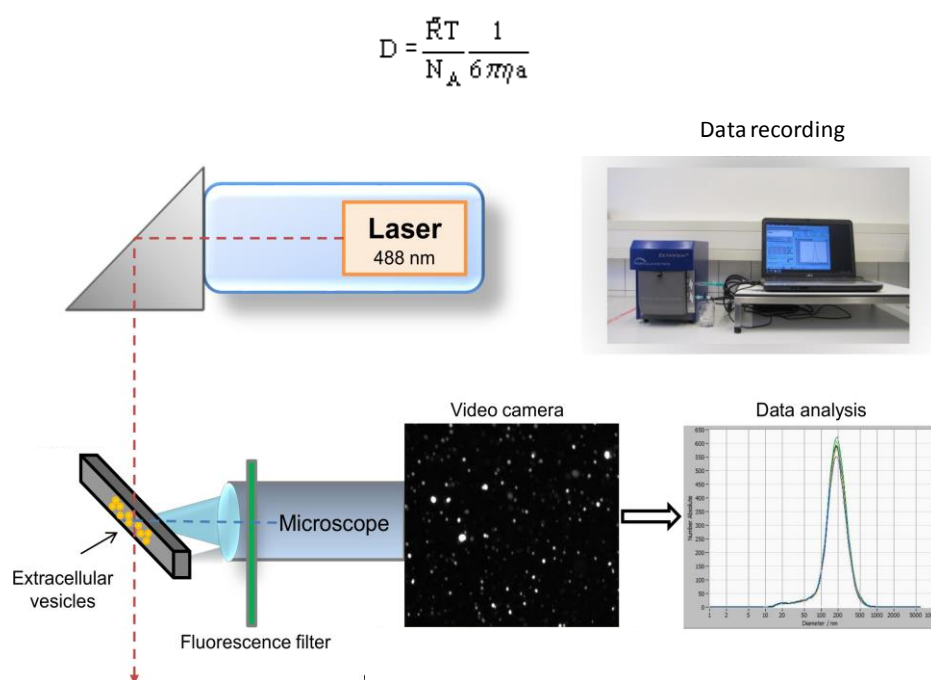


**Figure 7 Nano particle tracking system**

*(Adapted from* Weber et al., 2019)

*Electron microscope*

The use of the transmission electron microscope allows to directly observe isolated EVs (Rikkert et al., 2019). Sample preparation briefly consists of transferring droplets of a EVs suspension on metal grids which are then fixed with a 2% paraformaldehyde (PFA) solution. One disadvantage of this technique is that the image is selected by the operator, therefore it is just recommended as a tool to validate the presence of proper EVs in the analyzed sample by detecting the double lipid layer and approximate round shape. This technique should not be considered when the aim is to evaluate EVs quality characteristics such as if the sample is sufficiently pure (Rikkert et al., 2019). EVs isolation with the ExoQuick™ commercial kit interferes with the image quality, therefore using

this type of microscope to acquire images after this type of isolation is not recommended (Serrano-Pertierra et al., 2019). Staining of mesh grids is possible after fixation. This allows to label EVs with certain cell surface markers like CD9 and CD63. Labeling is usually performed using immunogold which are gold particles bound to secondary antibodies. Image acquisition by TEM shows immunogold positive staining with a dark color labeling (Klymiuk et al., 2019).

The cryo-electron microscopy (cryo-EM) is a methodology that consists of loading sample in a grid that is freezed by cryo-immobilization into a system device at low temperature (below -175°C). Automated devices such as Vitrobot (FEI) and Cryoplunge (Gatan) have been used to prepare the sample and to leave a vitrified layer of ice in the sample allowing it to have a better morphology and imaging at the time of acquisition (Cheng et al., 2015). The cryo-EM allows acquiring EVs in a more hydrated and native state when compared with the typical fixation (Ting et al., 2018). This methodology permits acquiring EVs with a more defined shape and to identify different EVs sizes. In addition to the computing image acquisition which provides a three-dimensional structure where EVs proteins can be identified. A limitation of cryo-EM is that it demands expertise training and it is time-consuming (Cheng et al., 2015).

*Flow cytometry*

The use of this technique has been suggested to study EVs and its use is reported in numerous articles (Welsh et al., 2017). The limitation in determining EVs size and count through flow cytometry is the requirement of precise standardization by the use of calibration beads with known diameter. Titration of dyes is also necessary to diminish background noise to ensures good quality detection (Welsh et al., 2017). Such a step is required due to the low fluorescence signal that each EV can produce. It can be achieved by multiples washings and centrifugations before EVs acquisition (Gray et al., 2015). However such additional steps are bound to reduce the number of EVs. EVs phenotyping by identification of cell surface markers is affected by their small diameter so that a very limited number of copies (tens of copies) for each marker can be found on their surface (Welsh et al., 2017). Flow cytometers are not able to analyze single EVs due to their size, this is because when EVs are ran at high concentrations flow cytometers have issues in discriminating single particles. Serial dilutions might help, but they would also consistently increase analysis time. Conventional flow cytometers are not capable of detecting 30nm EVs, most cytometers detect particles around 500nm. There are specialized cytometers that have a size resolution of 200nm that, along with calibration beads, might be used to analyze larger EVs (Szatanek et al., 2017). Currently, some instruments with higher sensitivity are being developed (Welsh et al., 2017). The use of antibodies and PKH lipophilic dyes to stain biological membranes in flow cytometry are commonly used to detect EVs presence (Gray et al., 2015). This PKH dye interacts with the lipid bilayer membrane of EVs and the labeling fluorescence is detected. PKH

dye is added to the EVs and incubated for minutes, followed by washings and centrifugation for further analysis by flow cytometry. The use of latex beads coated with monoclonal antibodies can pool multiple smaller (<100nm) EVs at the same time, allowing for easier EVs detection even on instruments with a worse size resolution (Szatanek et al., 2017).

## 4. Extracellular vesicles as a potential therapeutic treatment

The interest and relevance of EVs is that they might provide some advantages when compared to MSCs in therapy. For example, when MSCs are stimulated with specific cytokines (TNF-α and IFN-γ), MSCs release EVs with typical characteristics. EVs prove themselves to be stronger inhibitors of the cycloxigenase (COX2) and PGE2, which play a role as anti-inflammatory and immunomodulators, than MSCs (Harting et al., 2018). Moreover, when adding MSCs derived EVs to chondrocytes affected by pro-inflammatory cytokines, an increase in COX2 expression, cell proliferation, collagenase II and proteoglycan production has been observed. Moreover, by the effect of EVs a decrease in the pro-inflammatory NFkB, cytokines IL-1α, IL-6, IL-8, and IL-7 has been also suggested. These types of cytokines usually are key mediators during the onset and progression of OA (Vonk et al., 2018). Another advantage over MSCs is that EVs can be produced on a large scale because cells release a large amount of EVs into the media when cultured *in vitro* (Chen et al., 2011). For example, around $4.46 \times 10^9$ EVs/mL can be obtained from culture medium from bone marrow cells (Tracy et al., 2019a). In the case of obtaining specific EVs characteristics, a study modified human embryonic stem cells HuES9.E1 MSC with p21 to carry MYC gene. These transformed cells released EVs that when implanted for example in infracted hearth areas in mice have shown a lower size and improve hearth function (Chen et al., 2011). An important characteristic of EVs is that they can cross certain barriers like the endothelium of blood vessels due to their small size. A study showed that after the administration of EVs by intravenous injection in the jugular vein in mice a reduced influx of neutrophils in the bronchoalveolar fluid was evident (Zhu et al., 2014). Additionally, it was reported that EVs also possess the capacity to reduce senescence in cells. A recent study showed that MSCs treated with EVs MSCs showed a reduced expression of the enzyme beta-galactosidase and the gene expression of p21 and p53 associated with senescence. A reduction in the level of reactive oxygen species within the cells has been also found when adding EVs to cells that are going through senescence (Liu et al., 2019). Several studies have shown that by adding EVs in rodent cutaneous models, the healing of the scratch was accelerated (Cabral et al., 2018). In summary, most of the studies in EVs so far have been done *in vitro* but more are still needed to understand the basis of their performance *in vitro*. Furthermore, more studies would also be needed to confirm theirs *in vivo* performance and possible applications.

## 5. Extracellular vesicles content

The content of EVs depends on the type of cell from where they were released. This might be correlated with the cell phenotype and the metabolic state of the cells. For example, EVs-derived from stem cells can activate regenerative programs in other neighbor cells while EVs-derived from injured cells might activate differentiation programs (Tetta et al., 2012). EVs are known to carry protein, RNA and also lipid content. In the scientific media, a data-base named ExoCarta (http://www.exocarta.org) serves to update EVs knowledge from research. The updated database of protein content shows a total of 100 proteins that can be found in EVs. They are listed according to the frequency with which they have been identified. Some of the most commonly detected are: CD9, heat shock protein (HSPA8), programmed cell death 6 interacting protein (PDCD6IP), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and CD63. To date, there are around 4,000 entries for miRNAs and around 1,000 for lipid entries. This data-base is updated from a different type of publications from where EVs have been studied. The reports on EVs are not limited to proteins or surface receptors, there are also nucleic acids like RNAs, DNA and non-coding RNAs like microRNA and tRNA. Membrane proteins like tetraspanins (CD63, CD81, etc.) on EVs have been also recognized in EVs (Raposo and Stoorvogel, 2013). EVs are also composed of lipids like cholesterol, sphingomyelin, phosphatidylserine, ceramides which are important during cell communication (Thery et al., 2009 ;Raposo and Stoorvogel, 2013). EVs released by human adipose cells have were analyzed and the presence of CD63 and lysosome-associated membrane protein (LAMP2) was studied. After a microRNA analysis of this EVs fraction, data showed that miRNAs present in the EVs fraction were mainly related to the anti-inflammatory activity like miR-let 7 family which plays an important role during chronic inflammation (Mitchell et al., 2019). In equines, sequencing of derived EVs from MSCs adipose subcutaneous cells shown that the angiogenic pro-angiomiR-30 was the most present in EVs from equine adipose MSCs. In addition to this, miR-100 was also found in these EVs, which might be related to the modulation of vascular responses. Generally speaking, the most frequent type of RNA category found was the ribosomal RNA, followed by snoRNA (Capomaccio et al., 2019).

**General aims of the PhD project**

The use of MSCs for treating equine leg's pathologies has been largely recommended and studied. However, the knowledge of MSCs mechanisms of action in vivo still has some gaps that need to be filled. Therefore, one of the objectives of this Ph.D. project was to isolate MSCs from different tissue sources in order to evaluate their phenotypic characteristics and evaluate theirs in vitro performance. This was done in order to better understand and compare MSCs properties because their source influences their behavior.

As a follow-up, a subsequent objective was to determine that some of MSCs' effect on other cell types/tissues is carried out by extracellular vesicles. This last objective was to evaluate their effect of these EVs in an in vitro inflammatory model using chondrocytes which are one of the main affected cell types during osteoarthritis.

**Aim 1: Isolation of MSCs from different tissue sources**

**Material and methods**

*Tissue harvesting and isolation of cells*

Samples from three mixed-breed horses (10-16 months old) were collected at the slaughterhouse according to the Italian regulations under the supervision of the Veterinary Services of the Italian National Health Service, branch of the Ministry of Health. Tissue samples were obtained as follows: bone marrow (through the scraping of the bone marrow area of the sternum), synovial fluid (radiocarpal knee joint) and adipose tissue from mesenteric, neck and tail base fat regions. All sample tissues except synovial fluid were rinsed twice with PBS before the subsequent digestion step. BM tissue was washed with PBS and centrifuged at 1200g then filtered through a 40 µm mesh. Later, BM cells were cultured in proliferation medium (high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), 10 % fetal bovine serum (FBS) (Gibco), L-glutamine, amphotericin B and penicillin-streptomycin (all from Sigma-Aldrich). Tissue from different adipose areas was washed with PBS and cut it in small pieces and digested with 250U/mL collagenase type I (BD Bioscience) at 37°C during 3-4 hours. Then, adipose tissue was centrifuged at 1200g, filtered with a 40 µm mesh and cells were cultured in proliferation medium. Two ml of synovial fluid samples were gently diluted with 3mL proliferation medium and directly seeded in tissue culture dishes. The cells obtained from BM, synovial fluid and adipose tissue were all cultured in 100mm tissue culture dishes (Sarstedt). After 24 hours in culture, the medium was replaced with a fresh one to eliminate non-adherent cells. Cells were cultured at 37°C with 5% $CO_2$ in proliferation medium until confluence then split in new cell culture dishes.

*Characterization of cells isolated from different tissue sources*

In order to know if isolated cells showed the MSCs profile a characterization with cell surfaces markers was made. According to Dominici (Dominici et al., 2006a), MSCs identity should be confirmed if the isolated cells meet these criteria: adherence to plastic, expression of specific cell surface markers and tri-lineage differentiation capacity (osteocytes, chondrocytes and adipocytes differentiation). For this reason, at first, I evaluated cell phenotypes by flow cytometry. Cells were detached with 0.25% trypsin–0.02% EDTA (Sigma-Aldrich) then counted, washed with PBS and centrifuged at 500g for 5 minutes. Aliquots of 250,000 cells per tube were prepared and washed with PBS. Cells were then incubated at 4°C in the dark with CD11α/18-FITC (clone CVS9, anti-horse, 1:160) (BioRad), CD45-Alexa Fluor 488 (clone F10-89-4, anti-human, 1:160) (BioRad), CD79α-Alexa Fluor 488 (clone HM57, anti-human, 1:80) (BioRad), CD90-PE (clone Thy1-A1, anti-

human, 1:400) (R&D Systems), CD105 (clone SN6, anti-human 1:80) (BioRad) and MHC-II-FITC (clone CVS20, anti-horse, 1:80) (BioRad) antibodies for 15 minutes. The cells labelled with the CD105 antibody were then washed and incubated with an anti-mouse IgG Pacific blue-conjugated secondary antibody (1:500) (Invitrogen). Later, PBS was added to wash and tubes were centrifuged at 500g for 5 minutes at 4°C. Cells were re-suspended with PBS for further flow cytometry analysis. As control samples, horse whole blood served to establish acquisition parameters on each cell type to select the population of interest. For data analysis 20,000 events were collected using an Attune Cytometer (Life Technologies) equipped with a 405 nm and a 488 nm laser. The data obtained were analyzed with the Attune® Cytometric Software.

*Cell tri-lineage differentiation*

To evaluate equine mesenchymal cell adipogenic differentiation capacity, 5000 cells/well were plated in a 6-well dish (CytoOne, Starlab). The osteogenic differentiation capacity of cells was evaluated by seeding 15,000 cells/well in a 6-well dish (CytoOne, Starlab). For the chondrogenic differentiation, 5x105 cells were seeded in a 2mL Eppendorf tube with a round bottom to allow a three-dimension spontaneous cell aggregation. Differentiation StemPro® (Gibco) medium was used for adipogenesis, osteogenesis and chondrogenesis respectively according to manufacturer instructions. For all cell differentiation cultures, medium was replaced every two days and cells remained in culture for around 14-days in the case of adipogenesis and chondrogenesis, while for osteogenic differentiation two stages were tested: positivity to alkaline phosphatase (AP) activity (~7-days) and up to 21-days for final osteogenic differentiation (**Figure 8**).
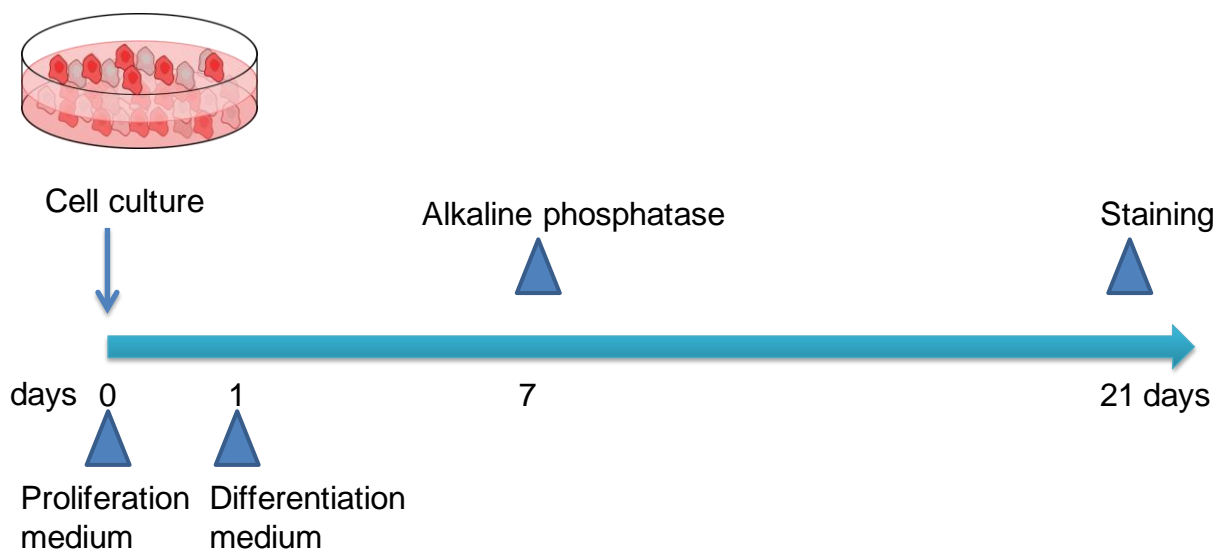


**Figure 8 Cell differentiation diagram treatment**. Cells were cultured and then differentiation media was added. Cells were analyzed at day 7th to evaluate the pre-osteogenesis differentiation by alkaline phosphatase. Final differentiation was evaluated at day 21th for all types of differentiation (osteogenesis, chondrogenesis and adipogenesis).

*Histological evaluation of cell tri-lineage differentiation*

For the differentiation staining, medium was removed from all cell types and they were washed with PBS carefully trying to not remove the cell layer from the wells and to maintain the sphere-like cell structure in the chondrogenic differentiation cultures.

Adipogenic culture cell samples were incubated for 1 hour with 4% formalin. Then, formalin was discarded and samples were washed twice with $ddH_2O$ water and 60% of isopropanol was added for 5 minutes. Cells were left to dry at room temperature. Then, sample cells were stained with Oil red O for 10 minutes. Oil red O was removed and four $ddH_2O$ washes were required before image acquisition with a Leica DMIL6000 microscope.

The staining for osteogenic differentiation consisted on cell incubation with 4% of formalin for 30 minutes. Cells were washed with $ddH_2O$ and then they were incubated with Alizarin red in darkness for 45 minutes. Subsequently, wells were washed again with $ddH_2O$ and viewed under the microscope.

Chondrogenic cells formed sphere-like structures which were washed carefully with PBS and then incubated overnight with 4% formalin. The next day, the sphere-like structures were incubated in 80% ethanol and then paraffin-embedded. Paraffin-embedded sections were cut with a 5µM thickness and mounted on superfrost slides (Thermo Scientific). The slides were de-paraffinized in Bio-Clear (Bio-Optica Milano), a xylene substitute, then washed in different ethanol solutions (100%, 90% and 60%) as described by Williams et al.1971. Slides were washed with $ddH_2O$ and later they were incubated in Mayers hematoxylin for 3 minutes and washed with tap water. An incubation with Safranin O was performed for 4 minutes. Slides' dehydratation was made by submerging them in solution with an increasing concentration of ethanol (50%, 70% and 100%). Then, slides were mounted with a mounting medium (EUKITT®) and images were taken after 24h.

*Cell migration*

Three replicate wells were set up for each different cell type (bone marrow, synovial fluid and adipose tissues) from each of the three horses. A total of 50000 cells/well were plated on top of 8 microns TC-inserts (Sarstedt) in 24-dish (CytoOne, Starlab) with proliferation medium. After 24 hours of incubation, medium was removed, then inserts were washed with PBS and the upper side of the membrane was cleaned carefully with a cotton swab. Inserts were fixed with 4% formalin for 20 minutes, after fixation they were washed with deionized water and the upper side of the membrane cleaned again with a cotton swab. Finally, inserts were incubated with 4'6-diaminide-2'-phenylindole dihydrochloride (DAPI) 1:2000(Sigma-Aldrich) and images were acquired with an inverted fluorescent microscope. An image of the entire lower side of the TC-insert membrane was

captured with a 10x objective using the LAS AF software and nuclei were counted with ImageJ software.

*Mesenchymal equine cells growth assessment*

To estimate cell growth, each cell type was seeded in a 6-well plate (CytoOne, Starlab) with proliferation medium at a density of 20,000 cells/well. The experiment was performed from each of the three horse's harvested tissue, and duplicates were made on each different type of tissue. Cells were incubated and cell numbers were assessed at 0, 24 and 48 hours. The medium was discarded and cells were fixed with methanol-acetone (1:1) which was subsequently replaced with Tris-HCl buffered saline (0.1M Tris-HCL, 8g NaCl, and pH 7.6). Cells were incubated with DAPI (Sigma-Aldrich) to stain nuclei and images were acquired at 10x magnification in five random areas per well with an inverted fluorescent microscope Leica AF6000 LX equipped with a Leica DFC350FX digital camera (Leica Microsystem, Wetlar, Germany). Images were analyzed with ImageJ software (1.48version) to automatically count the stained nuclei in each image. Cell growth was expressed as the ratio of nuclei counted at 24h or 48h and the count from a control well stained at time 0.

*Wound healing assay*

The wound healing assay (or scratch assay) consisted on creating a wound in a confluent cell monolayer, capturing the images and then measuring the area of the gap to quantify the filling rate at 16 hours. Endothelial cells harvested from equine carotid artery were kindly provided by Dr. Walid Azab from the Free University of Berlin (Freie Universität Berlin, Institut für Virologie Robert von Ostertag-Haus-Zentrum für Infektionsmedizin, Germany). Endothelial cells were seeded at a density of 150.000 per well in collagen-coated 24 well plates (CytoOne, Starlab) and cultured with DMEM supplemented with 20% FBS and 1% non-essential amino acids (aa) until confluence (usually 24 hours). When cells reached confluence, a scratch was made with a 1000µL pipette tip, cell monolayers were washed with PBS, and media was replaced with one suitable for the appropriate assay condition. Two conditions were set up each one in 3 replicate wells: co-culture with MSCs and MSCs conditioned medium. In the co-culture wells, 24.000 MSCs at passage 1 from each sampled area were seeded in individual transwell (Sarstedt TC-inserts with a 1-micron porous membrane to prevent cell migration through the membrane itself). Culture medium was switched to DMEM 2% FBS with 1% non-essential aa. As control, transwells prepared with 24.000 endothelial cells were used and processed at the same way. To test the effect of conditioned medium, MSCs were seeded at passage 0 until confluence. Culture medium was then changed to fresh DMEM 10% FBS. After 48 hours conditioned medium was collected and immediately added to the appropriate wells were the scratches on endothelial cells were already performed. For this condition, control was set up using 48h conditioned medium from endothelial cells (DMEM 10%

FBS). Images were acquired with a 10x objective at the time the scratches were performed (time 0) and after 16 hours in culture. Three adjacent areas were photographed per each well (**Figure 9**). The images were then analysed by Image J software with the MRI Wound Healing Tool to calculate wound areas. Wound areas at 16h were normalized to their respective time 0 measurement and therefore data are presented as a ratio.

Cell culture:
✓ Endothelial cells and MSC types (tissue sources)
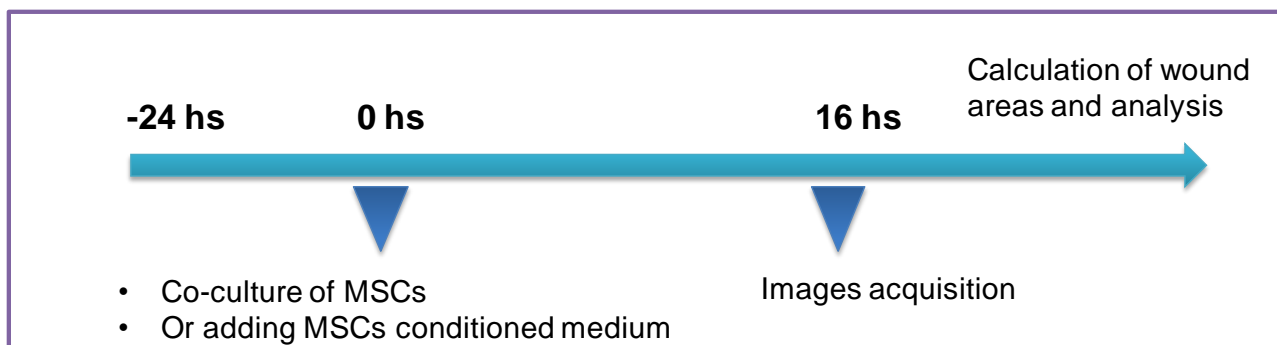✓ Endothelial cells and MSC conditioned medium



**Figure 9 Wound healing assay.** Endothelial cells were grown until confluence. A scratch was made by a pipette tip. Two conditions were used: 1) A co-culture with MSCs and 2) Adding conditioned media. Cells were added at time 0 and images were acquired after 16 hours to observe the closing of the wound area.

*Statistical Analysis*

Flow cytometry data are expressed in percentages as mean ± standard deviation and this data corresponds to the frequency of the positive cells in the analyzed population. For cell growth and results normalization was made and differences among the different tissue sources were estimated by a non-parametric test Kruskal-Wallis followed by a *post-hoc* pairwise comparison Wilcoxon-Mann-Whitney test. As for the migration test data was not normalized as they were absolute cell counts but the seeded cell amount number was the same in all samples. Wound healing data was normalized and statistical analysis was performed by a Kruskal-Wallis independent sample test followed by a *post-hoc* pairwise comparison Wilcoxon-Mann-Whitney test. A p-value of <0.05 was considered statistically significantly different. The statistical analyses were performed by the aid of SPSS statistics version 24 (IBM SPSS Statistics®, USA).

**Aim 2: Isolation of EVs from different types of MSCs**

**Material and methods**

*Setting up of MSCs cultured with EVs free FBS (Tendon Biology group at the Royal Veterinary College (RVC), United Kingdom)*

To obtain an EV-free FBS, FBS was ultra-centrifuged before being added into this media. FBS was prepared by an overnight (18 hours) ultracentrifugation (100,000g speed) at 4°C without break using a Beckmann centrifuge at the Camden, London campus of the Royal Veterinary College. Ultra-centrifuged FBS was further filtered (0.2μm) to eliminate remaining exogenous EVs.

Bone marrow cells were seeded (100,000 cells/well) for a population doubling (PD) study that allowed me to evaluate cell performance under four different culture conditions: 1) Normal FBS with DMEM (D10), 2) Pellet obtained after the FBS high-speed ultracentrifugation diluted with PBS + DMEM, 3) Exo-free ultra-centrifuged FBS + DMEM and 4) Commercial Exo-free FBS (Exosome-depleted fetal bovine serum qualified one shot™ Gibco) + DMEM. Cells were seeded and cultured using the four conditioned media with their corresponding FBS for 13 days (**Figure 10**). Cell number was counted with an automated cell counter (Countes® II FL, Thermofisher) and evaluated and cells were counted at 5 different days: 24 hours, 3 days, 6 days, 10 days and 13 days after being under each different media conditions. Images were acquired at different objectives (10x). Cell count was analyzed using the formula PD= ln $(N/N_0)$/ln2 (adapted from Schwarz et al., 2012).
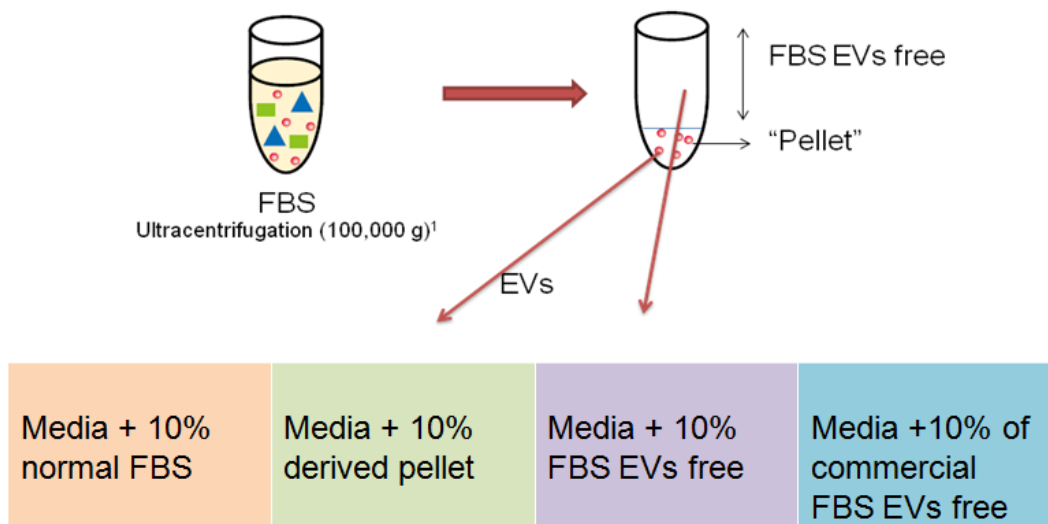


**Figure 10 Diagram of the different type of FBS on bone marrow equine cells**

*Culture of MSCs*

Equine MSCs were isolated from tissue and cultured in 100mm diameter dish (Sarstedt) in proliferation media composed of DMEM 1X (Sigma-Aldrich), 10% free EVs FBS (Gibco), 4.5g/L D-Glucose, 2 mM L-Glutamine, 2.5μg/mL amphotericin B and 1% penicillin-streptomycin (all from Sigma-Aldrich). When MSCs cultured in proliferation medium reached 80% confluence, the medium was replaced with fresh EV free medium. After 24 hours, the medium was collected for further analysis. MSCs were cultured at 37°C and 5% $CO_2$.

*Isolation of EVs from a different type of MSCs*

Approximately 5mL of medium was recovered from MSCs cultures and a further wash of the cultured monolayer was done with 5 ml of PBS. Such PBS was then added to the collected medium. Samples were centrifuged at 259x*g* for 5 minutes at 4°C in a refrigerated centrifuge (Eppendorf 5804R, rotor A-4-44). The supernatant was then transferred to a clean tube and centrifuged at 3000 rpm for 5 minutes at 4°C. The collected supernatant was filtered (0.2 μm) and placed in polyallomer conical tubes. Ultracentrifugation at 100,000g was performed for one hour at 4°C (L8-70M Beckman L8M class 7, Rotor SW4ITI) with a no-break deceleration. The resulting pellet was recovered in 500 μL of PBS (Sigma-Aldrich) for further analysis. Samples were used fresh or frozen at -80°C with 1% of dimethyl sulfoxide (DMSO, Sigma-Aldrich).

*Nanoparticle analysis*

Samples were diluted (1:200) with PBS before the acquisition. They were analyzed with a Nanosight LM10 (NanoSight Ltd) equipped with a 405 nm laser and a microscope Hamamatsu digital camera C11440 (ORCA Flash 2.8) at a 20x magnification. Particle concentration and size were acquired three times in motion during a 30 seconds video. Data was generated by the nanoparticle tracking analysis NTA 3.1 software. Each MSCs type of media from each horse was analyzed three times.

*Transmission electron microscope (TEM)*

Isolated EVs were placed on coated grids (Pioloform® film 200 Mesh Cu, Agar Scientific) and fixed with 1.25% glutaraldehyde 1% paraformaldehyde in 0.1 M Sorensen's phosphate buffer for 15 minutes. Washings were done with Sorensen's phosphate buffer five times for 10 minutes and excess liquid was dried on Whatman® filter paper. Grids were dried at room temperature before the analysis. Immunogold staining consisted of washings with tris-buffered saline (TBS) followed by incubation with goat serum (10%) + immunogold tampon for 1 hour. Subsequently, a two-night incubation with primary antibody CD9 (1:10, Clone HI98a, anti-mouse) at room temperature was performed. After that, a 1-hour incubation with secondary antibody anti-mouse IgG immunogold (EM. GAM10, BB inter) was done. Three 15 minutes wash with PBS were done and then a fixation in 2.5% glutaraldehyde for 10 minutes. Other three 15 minutes wash with PBS were performed

followed by overnight sample drying at room temperature before image acquisition. A transmission electronic microscope JE1-1010 JEOL was used to observe grids at a 250k magnification. Image acquisition was made with soft imaging system analysis® (Megaview III soft imaging system) software.

*Statistical analysis*

Different media culture conditions for the population doubling time were evaluated by analyzing the cell counts on the bone marrow cells. A Kruskal-Wallis independent sample test was used to test for differences among treatments. Differences in the size of isolated EVs and concentration was evaluated using a Kruskal-Wallis test as well. Differences were considered as statistically significant for *p*-values < 0.05. All the results were analyzed using IBM SPSS software version 24.

**Aim 3: Evaluation of the effect of EVs in an inflammatory model**

**Material and methods**

Chondrocyte cells were kindly provided by Dr. Elisabetta Chiaradia from the Department of Veterinary Medicine, University of Perugia, Italy. Chondrocytes cells used in this experiment were isolated from young horses from the metacarpo/metatarsophalangeal joint (Mancini et al., 2017). Chondrocytes used in this experiment were from passages 1-3. Equine chondrocytes were seeded (300,000 cells/well) in a 6-well plate (CytoOne, Starlab) with proliferation media (DMEM 1X + 10% free EVs FBS, 4.5 g/l D-Glucose, 2mM L-Glutamine, 2.5 µg/ml amphotericin B and 1% penicillin-streptomycin, all from Sigma-Aldrich). Chondrocytes were then starved for 24 hours with DMEM + 2% bovine serum albumin (BSA) (Sigma-Aldrich). After this time, treatments started (**Figure 11**) and consisted in: 10ng/mL of interleukine-1 beta (IL-1β) alone, 10ng/mL of tumor necrosis alpha (TNFα) alone and the cytokines along with purified EVs (13333 EVs/cell)(amount of EVs/cells adapted from Collino et al., 2017) derived from the different type of MSCs. Later, chondrocytes were processed for RNA extraction and further gene expression analysis. Each treatment was performed in duplicates using each EVs-derived from different types of MSCs (bone marrow, synovial fluid and adipose cells from neck fat tissue).
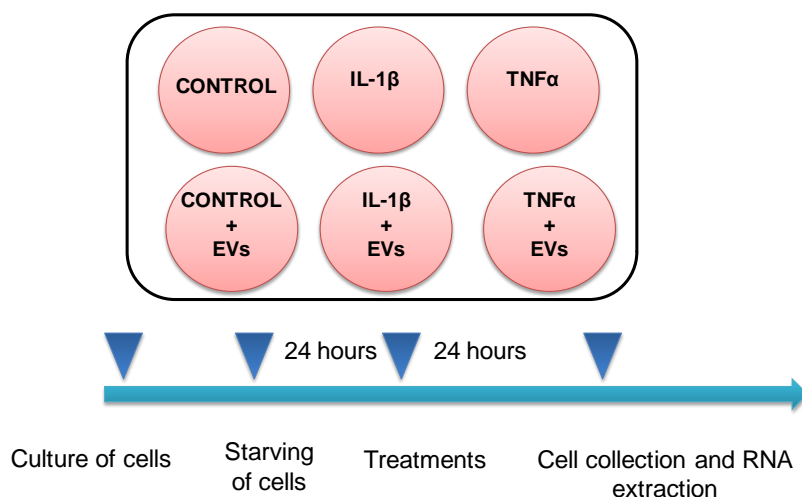
**Figure 11 Experimental design of chondrocytes treatment with cytokines and EVs**

*Gene expression*

Chondrocyte cells were lysed with TRI Reagent® (Sigma-Aldrich) and RNA was extracted following the manufacturer's recommendations. RNA was measured with a spectrophotometer (Nanodrop 2000, Thermofisher). Samples reverse transcription to cDNA was made using iScript™ RT mix using an iCycler (Bio-Rad). Real-time polymerase chain reaction (PCR) was performed with Sso Advanced™Universal SYBR Green Supermix using a CFX Connect Real-time System (Bio-Rad) under the following conditions: cycles were 95°C for 3 minutes, 95°C for 10 seconds, then 62°C for 30 seconds, 95°C for 10 seconds and a melt curve of 65°C with an increment of 0.5°C for 0.5 seconds.40 cycles were performed per each reaction. Data was obtained using BioRad CFX Manager 3.1 software. Equine specific primers (*Equus caballus, all from Eurofins Genomics*) were designed and used (**Table 3**): interleukin 6 (*IL-6*); A disintegrin-like and metalloprotease with thrombospondin (*ADAMTS*) type 4 and 5; tissue inhibitor of metalloproteinases (*TIMP*) 1,2 and 3; matrix metalloproteinases (MMP)1, 2, 3 and 13; aggrecan (*ACAN*); transcription factor Sox9; collagen type I (*COL1*) and housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). Relative gene expression was normalized with HPRT and calculated by the 2ΔΔCT method (Livak and Schmittgen, 2001).

**Table 3 List of primers used for the gene expression in chondrocyte cells**

| Gene | Forward | Reverse |
|---|---|---|
| IL-6 | GGCTACTGCTTTCCCCACC | CCCAGATTGGAAGCATCCGT |
| ADAMTS-4 | CATGTGCAACGTCAAGGCTC | AGTCACCACCAAGCTGACA |
| ADAMTS-5 | GAGATGACCATGAGGAGCACTAC | GGCCATCGTCTTCAATCACAG |
| TIMP-1 | ATCCCCTGCAAACTGCAGAGT | GCCCTTGTCAGAGCCTGTGA |
| TIMP-2 | AGAGTTGTTGAAAGTCGACAAGCA | ACCGAGCGATCACTCAGGAA |
| TIMP-3 | GACGCCTTCTGCAACTCTGA | GTACTGCACATGGGGCATCT |
| MMP-1 | AGGAGCCCAGTCGTTGAAAA | GTTCCCTTCGGTGAGGACAA |
| MMP-2 | TGAGCTCCCGGAAAAGATCG | AAAGGCAGCATCCACTCGTT |
| MMP-3 | GCAAGGGACGAGGATAGCAA | GTCTCATTTCTTTTCCAAGGTCGTAGT |
| MMP-13 | ACAAGCAGTTCCAAAGGCTAC | CTCGAAGACTGGTGATGGCA |
| ACAN | ACAACAATGCCCAAGACTAC | GCCAGTTGTCAAATTGCAAG |
| Sox9 | ACGCCGAGCTCAGCAAGA | CGCTTCTCGCTCTCGTTCA |
| COL1 | TGAAACTCTGC CACCCTGAATG | TTGTCCTTGCTCTTGCTGCTC |
| HPRT | TGA CAC TGG CAA AAC AAT GCA | 5'GGT CCT TTT CAC CAG CAA GCT |

*Statistical analysis*

Results derived from the real-time PCR analysis were evaluated first with a Shapiro-Wilk test to evaluate normality in the data. Samples that were normal were evaluated with a two-way analysis of variance (ANOVA). Samples that were not normal were analyzed with a non-parametric Kruskal-Wallis test. If the data was statistically significant ($p < 0.05$), a *post hoc* test was used for parametric using a Bonferroni as a *post hoc* test and non-parametric Wilcoxon-Mann-Whitney analysis. Each condition was evaluated against each other and the control (CTRL) served to compare in the treatments. All the results were analyzed using an IBM SPSS software 24 version.

**Results**

**Aim 1: Isolation of MSCs from different tissue sources**

Cells isolated from a different type of tissues (bone marrow, synovial fluid and adipose (neck, mesenteric and tail) were analyzed to verify their MSCs identity. As mentioned in the materials and methods section we followed the minimal criteria established for MSCs.

*Cell isolation and flow cytometry analysis*

Cells were grown in proliferation medium until confluence to be further analyzed by flow cytometry. Cells were between 1st and 3rd passage at the moment of the analysis (**Figure 12**).
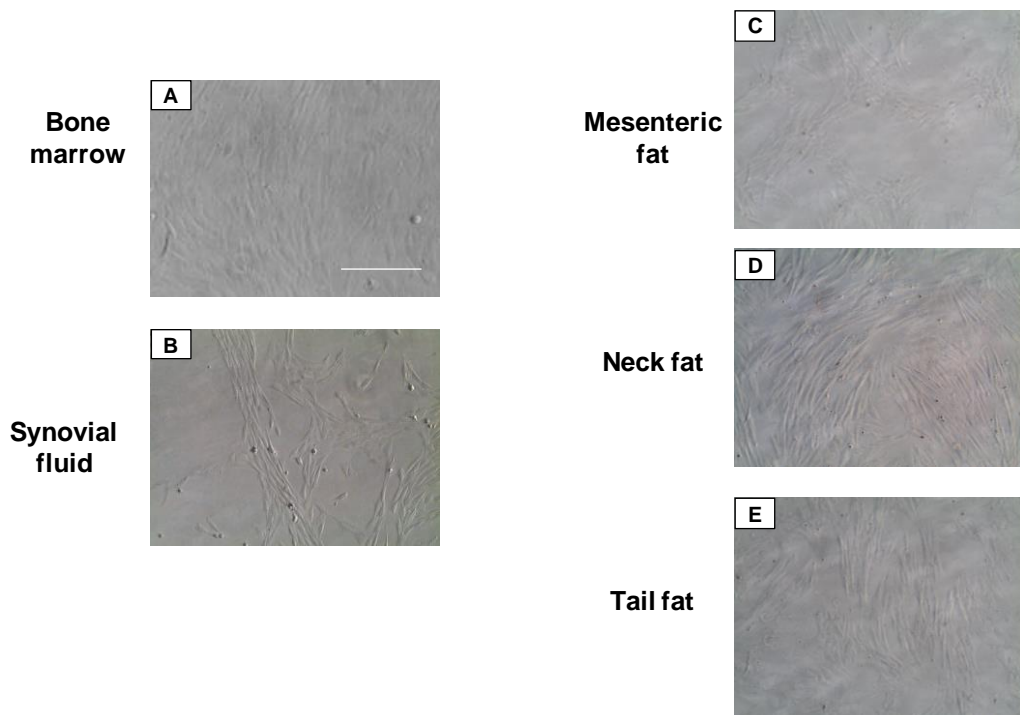
**Bone marrow** (A)

**Synovial fluid** (B)

**Mesenteric fat** (C)

**Neck fat** (D)

**Tail fat** (E)

**Figure 12 Cells isolated from different tissue sources after a week *in vitro* culture.** Cells cultured in proliferation medium. Typical spindle shape morphology is observed in all the isolated cells. Images are shown in a 20x magnification. Scale bar is 100 μm.

To identify mesenchymal stem cells, cultured cells were analyzed by flow cytometry using cell surface markers CD11α/CD18, CD45, CD79α, CD90, CD105 and MHC Class II described in MSCs literature (De Schauwer et al., 2011; Dominici et al., 2006). According to the acceptance criteria defined in literature for human MSCs, isolated MSCs should be more than 95% positive for CD105 and CD90, while less than 2% should be positive to the other tested markers. Representative plots of all markers tested are shown in **Figure 13** for all tissue sources. Two curves can be found in each plot, one being the unstained and the other one the cells incubated with the appropriate antibody.
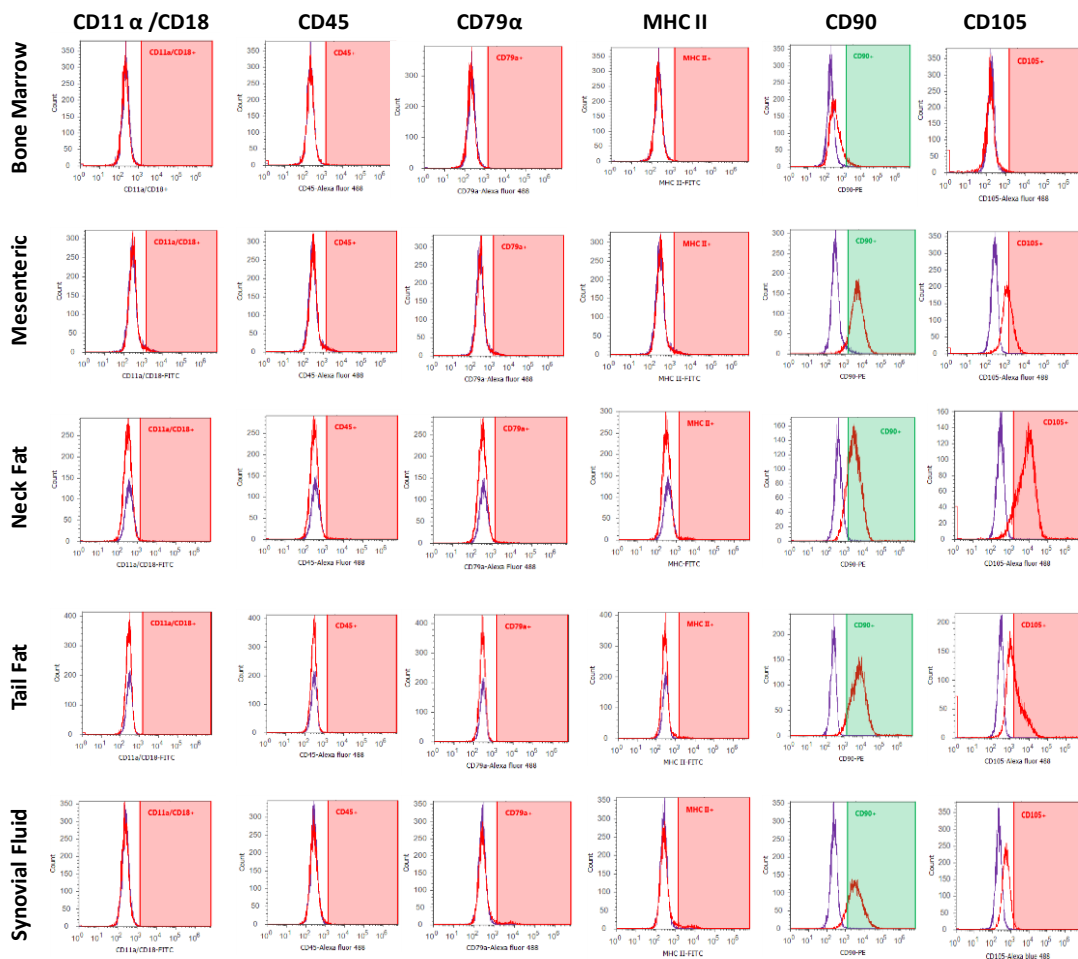
**Figure 13 Histogram graphic results obtained after the flow cytometry analysis. On the** left side of each graphic, the negative populations can be found. Inside the right gate, the positive populations are identified (red and green rectangle).

**Table 4** shows results with percentages of positive cells with their respective standard deviation. After the flow cytometry analysis, we could observe that the cells obtained from BM expressed the least CD90 when compared with synovial and adipose tissue cells. Most of the adipose tissue cells showed the highest expression of CD105 when compared with BM and synovial fluid cells. Moreover, CD90 was also highly expressed in all three adipose tissue cells. In the case of tail fat cells, they showed the highest CD90 expression, while neck fat presented the highest expression of CD105 of all the three adipose tissue cells. CD90 and CD105 expression showed higher variability. The frequency of positive cells ranged from 9% for CD90 and 13% for CD105 up to 97.043% and 84.385% respectively.

**Table 4 Expression of cell surface markers CD90 and CD105**

| | CD90 | CD105 |
|---|---|---|
| **Bone Marrow** | 9.132 (3.745) | 13.220 (9.813) |
| **Synovial Fluid** | 79.842 (24.734) | 16.229 (10.152) |
| **Mesenteric Fat** | 60.791 (30.878) | 34.129 (28,497) |
| **Neck Fat** | 84.631 (14.893) | 84.285 (8.541) |
| **Tail Fat** | 97.043 (1.981) | 55.904 (21.690) |

Data (n=3) is presented as mean percentages of positive cells and in the parenthesis (SD)

Positive cell surface markers in cells are reported in **Table 4** and the negative ones are in **Table 5.** All markers were negatively expressed with the except for CD45 and MHC II in synovial fluid cells where the frequency of expression was over 2%.

**Table 5 Expression of cell surface markers CD11α/CD18, CD45, MHC II and CD79α**

| | CD11α/CD18 | CD45 | MHC II | CD79α |
|---|---|---|---|---|
| **Bone Marrow** | 0.299 (0.179) | 1.196 (1.243) | 1.399 (1.788) | 0.539 (0.331) |
| **Synovial Fluid** | 1.566 (2.232) | 2.033 (2.066) | 3.164 (2.080) | 1.674 (2.213) |
| **Mesenteric Fat** | 0.133 (0.064) | 0.219 (0.083) | 0.336 (0.184) | 0.194 (0.118) |
| **Neck Fat** | 0.253 (0.111) | 0.696 (0.119) | 1.228 (0.687) | 0.648 (0.342) |
| **Tail Fat** | 0.150 (0.047) | 0.295 (0.050) | 1.656 (1.012) | 0.326 (0.172) |

Data (n=3) is presented as mean percentages of positive cells and in the parenthesis (SD)

*Cell differentiation*

Cells were cultured with differentiation media with the objective to evaluate their capacity to go through tri-lineage (adipogenesis, chondrogenesis and osteogenesis) differentiation. This is a characteristic expected when obtaining MSCs. Adipogenic differentiation was validated by Oil red O staining after 14 days in culture.
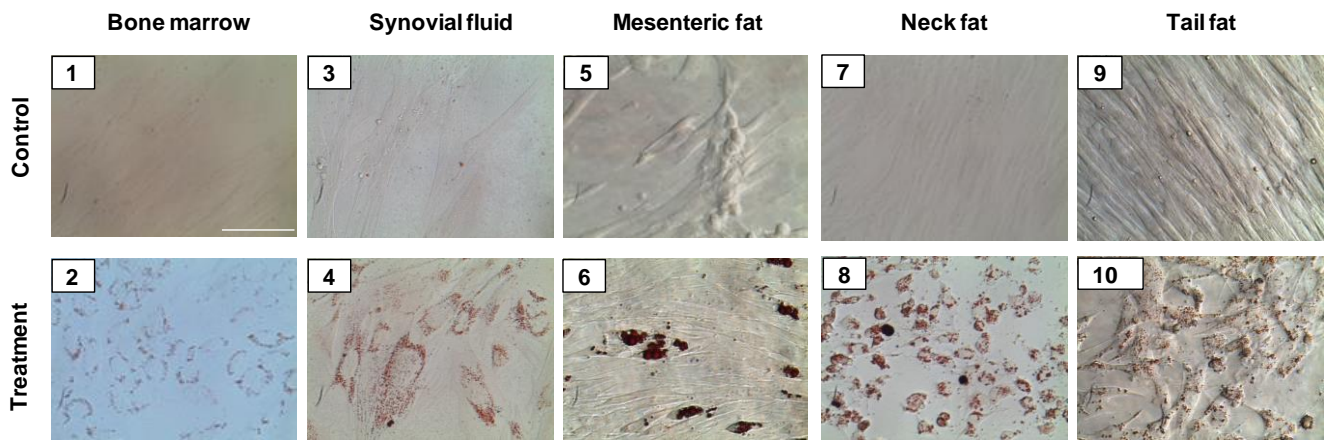
**Figure 14 Cells cultured in adipogenic differentiation media.** MSCs cultured in normal proliferation medium are shown in the upper row after Oil red O staining. MSCs that were cultured in an adipogenic differentiation medium are shown in the bottom row and were stained with Oil red O. Red areas indicate presence of lipid droplets.

The Oil red O staining is specific for lipid droplets within the cells that underwent adipogenic differentiation in treated cells. In all the analyzed cells and samples, cells were able to undergo adipogenic differentiation as showed by positive/red staining. Overall, adipose-derived cells showed more intense staining when compared to BM or synovial fluid-derived cells. Mesenteric cells (**Figure 14.6**) differentiation is more evident and showed the presence of typical intracellular lipid droplets.

For the chondrogenesis differentiation, a micromass was formed through cell aggregation (the pellet was formed almost right away after seeded and its size increased until the 14$^{th}$ day). After seeding the cells in 1.5 ml test tube, they rapidly aggregated. The pellet size of the chondrogenic treated cells was larger (images not show) in comparison with the controls. The control pellets did not show a defined structure and a basal Safranin O staining (**Figure 15**) was observed in the synovial mesenteric and tail controls (**Figure 15.3, 15.5 and 15.9**). Pellets formed by cells undergoing chondrogenesis were positive for Safranin O staining demonstrating the presence of proteoglycans (**bottom row in figure 15**) and also showed more defined nuclei structures when compared to controls.
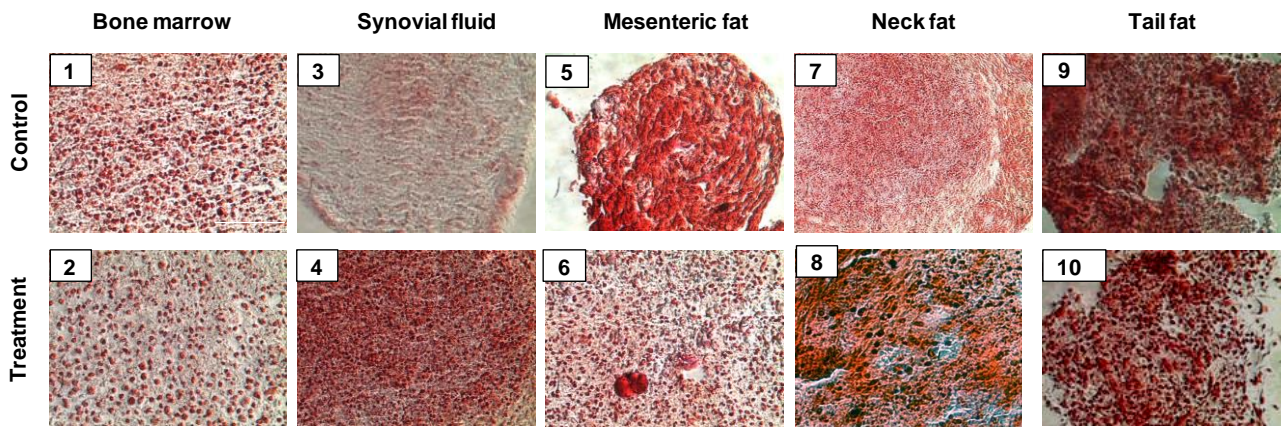
|  | Bone marrow | Synovial fluid | Mesenteric fat | Neck fat | Tail fat |
|---|---|---|---|---|---|
| Control | 1 | 3 | 5 | 7 | 9 |
| Treatment | 2 | 4 | 6 | 8 | 10 |

**Figure 15 Cells cultured with chondrogenic differentiation media.** Pellets stained with red Safranin O. MSCs cultured in proliferation medium are shown in the upper row, while MSCs cultured in a chondrogenic differentiation medium are shown in the bottom row. A more defined structure is shown in the cells cultured in differentiation medium.

Osteogenesis pre-differentiation was evaluated by AP staining which allows evaluating mineralization deposition in cells during osteogenesis (McNamara, 2012). All the controls in the proliferation medium were not stained whereas positive staining for MSCs cultured in osteogenic differentiation medium was detected demonstrating a pre-osteogenic differentiation in all the different cell types (**Figure 16A**). Final osteogenic differentiation was detected by Alizarin red staining which allows staining calcium deposits. Also, treated cells showed an irregular shape after 21 days in culture. As shown in **figure 16B**, in all the osteogenesis induced cell types, positive red-stained areas were detected, confirming final osteogenesis by the presence of calcium deposits.
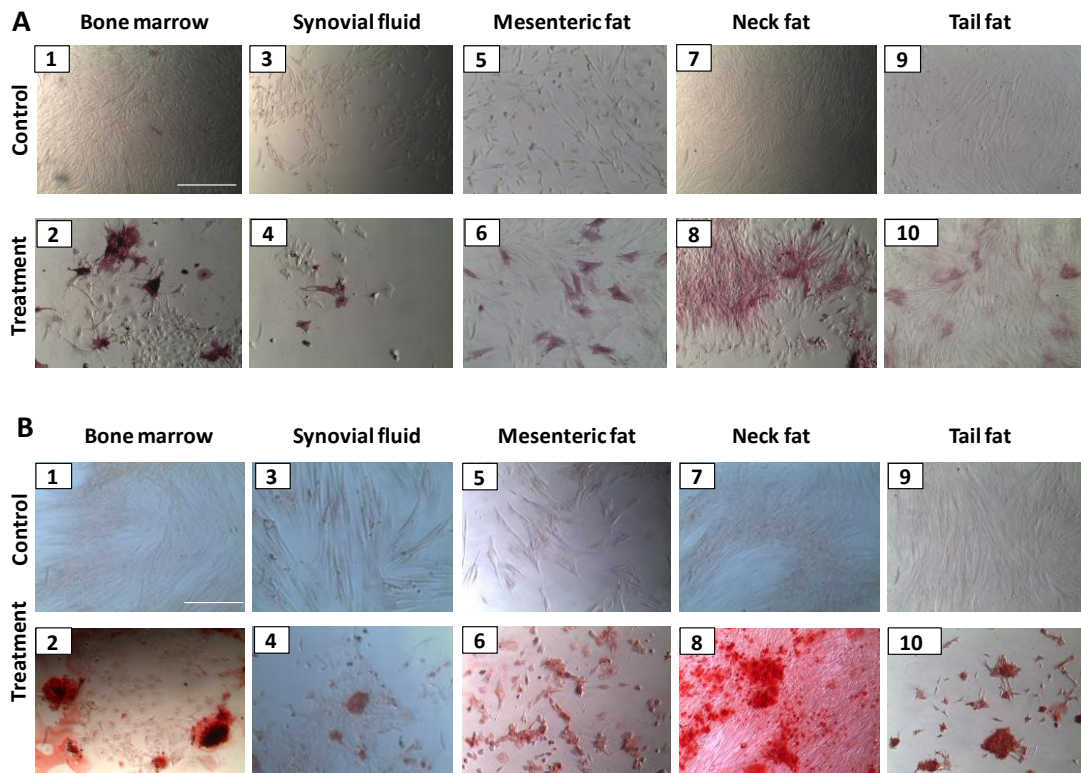
**Figure 16 Pre-osteogenic and osteogenic differentiation in MSCs.** The alkaline phosphatase staining is shown in A. Positive staining is shown in treatments as a red staining. Alizarin red staining is shown in B. Positive stained cells are shown in red colour in treatments. Images were obtained with a 10x objective (scale bar is 250 µm).

**The cell *in vitro* performance evaluation of cells isolated from the different bone marrow, synovial and adipose cells**

*Cell migration*

This migration assay might give us information on the ability of cells to move through a porous membrane which could be related to their attitude to migrate *in vivo*. The cell migration test was performed on all cells by the use of porous membrane inserts (transwells). At 24 hours, we could observe that there were significant (p<0.05) differences in the migration capacity of BM against mesenteric and neck fat cells (**Figure 17**). Within the adipose tissue cells, tail fat showed a higher tendency to migrate when compared with mesenteric and neck fat; however, migration was not statistically different (p>0.05) among cells recovered from the different adipose tissues (**Figure 17**). Tail fat, BM and synovial fluid were not significantly different and mesenteric and neck fat cells seem to be likely similar on their migration capacity. Synovial fluid cells showed a statistically significant higher migration capacity (p<0.05) than mesenteric and neck fat cells.
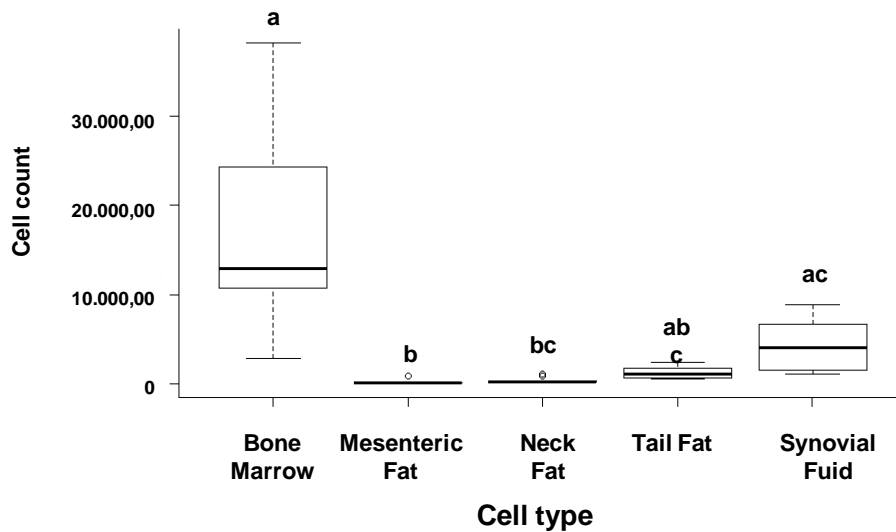
**Figure 17 Cell migration of different type of cells.** Graphs represent cell count results after the cell migration assay. BM-derived cells showed the highest migration capacity followed by synovial fluid derived-cells. *Letters indicate the results of post-hoc pairwise comparison. Groups identified with different letters are statistically different (p<0.05)*

*Cell growth*

Another parameter that might be important for the characterization of the MSCs and in particular to assess when considering different tissue sources is their proliferation capacity. Therefore cell growth was analyzed in vitro. Cells were plated in 6-well dishes and were evaluated at two different times (24 and 48 hours). At 24 and 48 hours in culture (**Figure 18**) synovial fluid cells were significantly different (p<0.05) against all the treatments.

When the analysis was repeated at 48 hours, we could observe that BM and neck fat cells were significantly different (p<0.05) from mesenteric, tail fat and synovial fluid cells (Figure 5). Among the adipose tissues, neck fat-derived cells were the ones statistically different (p<0.05) from the other two (**Figure 18**). Finally, synovial fluid seems to grow significantly faster (p<0.05) than any other cell type (**Figure 18**). Cell culture length seems to modify cell growth in all samples. Adipose cells growth patterns from mesenteric cells become significantly different (p<0.05) from BM cells after 48 hours in culture (**Figure 18**). In both cases, synovial fluid cells remain significantly different within both 48 and 24 hours in culture, presenting the highest cell growth capacity in *in vitro* cultures.
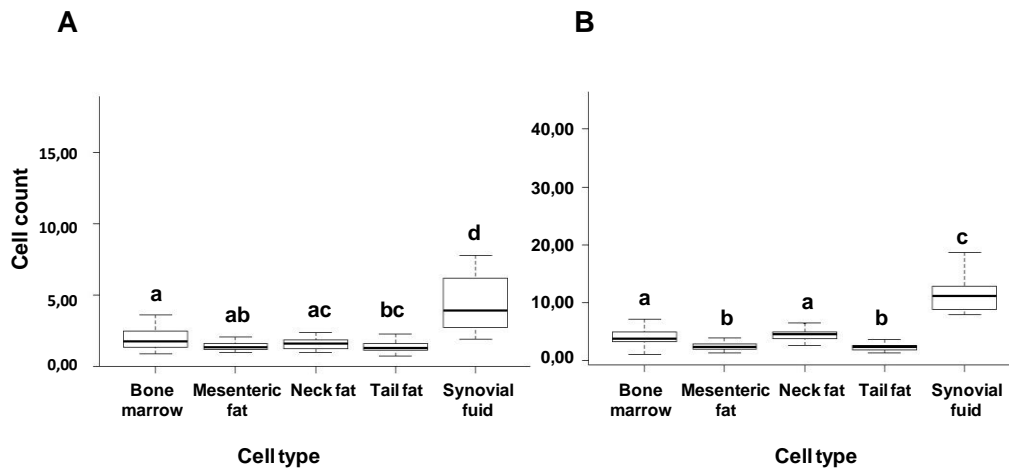
**Figure 18 Cell growth of different types of cells.** Left side (A) shows cell growth after 24 hours in culture. B) Shows cell growth after 48 hours in culture. *Letters indicate the results of post-hoc pairwise comparison. Groups identified with different letters are statistically different (p<0.05)*

*Wound healing assay*

MSCs-derived from BM, synovial fluid and adipose tissues were seeded in a co-culture with endothelial cells to analyze their ability to improve the wound healing capacity of a target cell population by closing a scratch area. The area of the scratch was evaluated at different time points (0 hours and after 16h) to calculate a ratio that expressed the closure of the gap. After 16 hours of being in the culture, differences in the amount of cells that were able to migrate to the wound area was analyzed. We could observe that the different types of MSCs showed no statistically significant differences (p<0.05) from the controls (endothelial cells) when they were seeded along with the endothelial cells (**Figure 20**). However, when conditioned medium from MSCs was used a statistically significant difference (p<0.05) was observed as the BM cells were the only ones to improve scratch filling (**Figure 21**). BM cells were the cells that were able to largely recover wound areas when compared with another type of MSCs. Representative images at time 0 and 16h are shown (**Figure 19**).
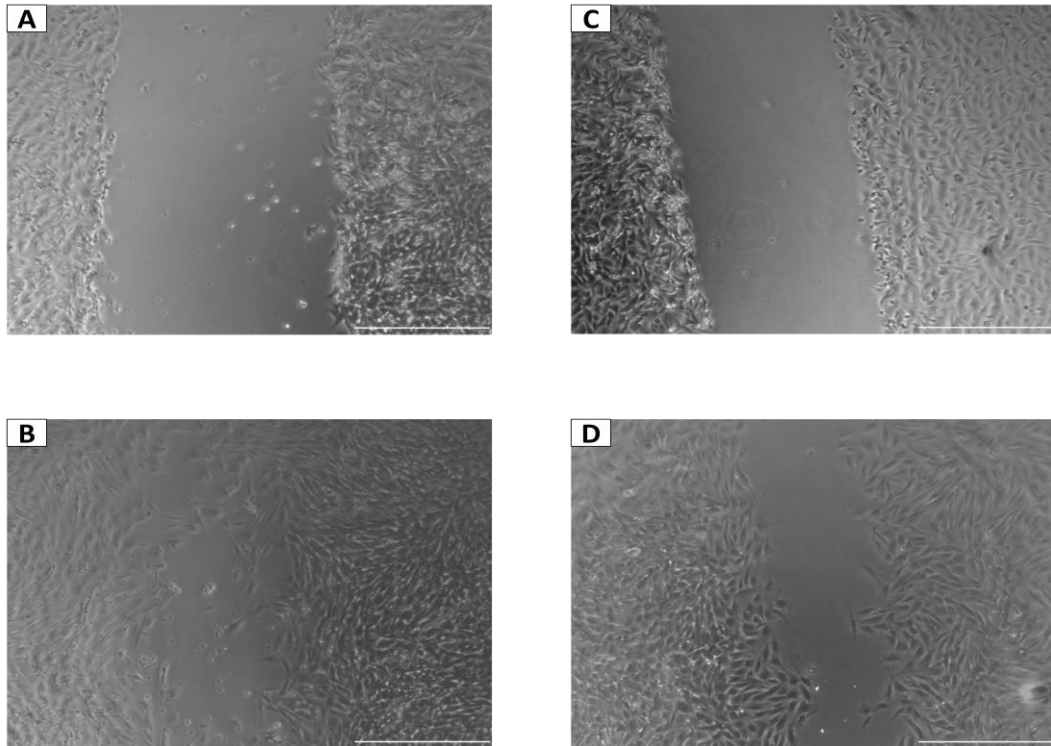
**Figure 19 Endothelial cells on a monolayer culture in the wound healing** assay A: Endothelial cells on a monolayer culture at time 0. B: Same scratch area of A after 16 hrs of culture with bone marrow cells conditioned medium. C: Endothelial cells monolayer at time 0. D: Same scratch area of C after 16 hours in culture with endothelial cells conditioned medium. Images were acquired with a 5x objective (bar 500 µm).
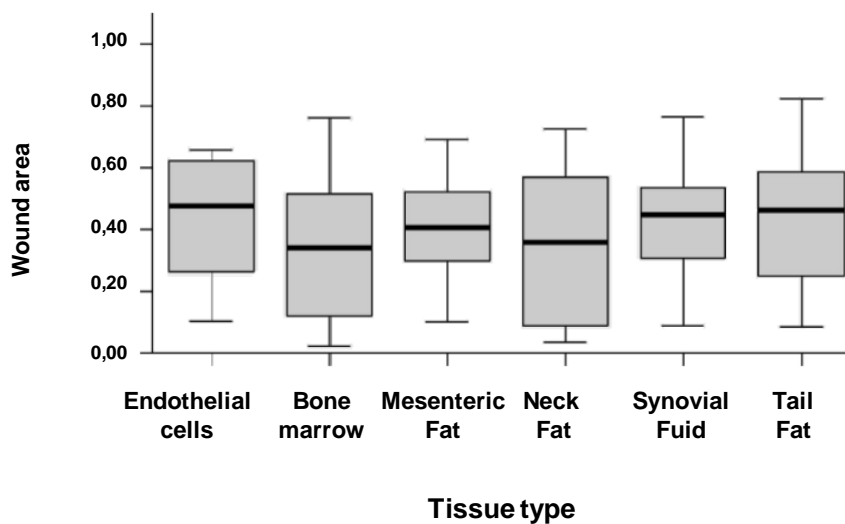


**Figure 20 Wound healing results from the co-culture condition.** Graphs represent results from the co-culture condition of the controls (endothelial cells added in the transwells) and every type of MSCs.
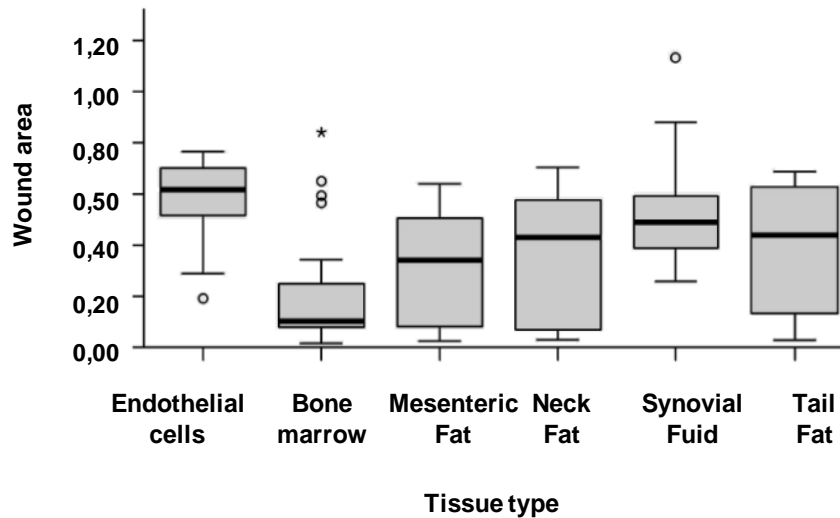
**Figure 21 Wound healing results using conditioned media.** Graphs endothelial cells (as control) and the different types of MSCs. Significant differences are represented by an asterisk (*) and were considered different at (p<0.05).

**Aim 2: Isolation of EVs from different types of MSCs**

In order to isolate extracellular vesicles from cultured MSCs a setup of the appropriate culture conditions needed to be performed. As commercially available FBS is a source of exogenous EVs, such serum is unsuitable for culturing MSCs with the purpose of isolating the EVs that they produce. In order to evaluate and choose culture conditions that were suitable for the isolation of EVs, an *in vitro* assay to evaluate MSCs proliferation was performed with different media. The cells used for such assay were BM-derived MSCs. Four conditions were tested using DMEM supplemented with a commercially available EVs free FBS (Gibco) (1), with regular FBS (2), with ultracentrifuged regular FBS (3) and with an equivalent amount of the pellet from the ultracentrifugation of the regular FBS (4). Population doubling was calculated for the in vitro culture no statistically significant (p>0.05) (**Figure 22**) difference was found among the treatments at all time points analyzed ($1^{st}$, $3^{rd}$, $6^{th}$ and $10^{th}$ day of culture).
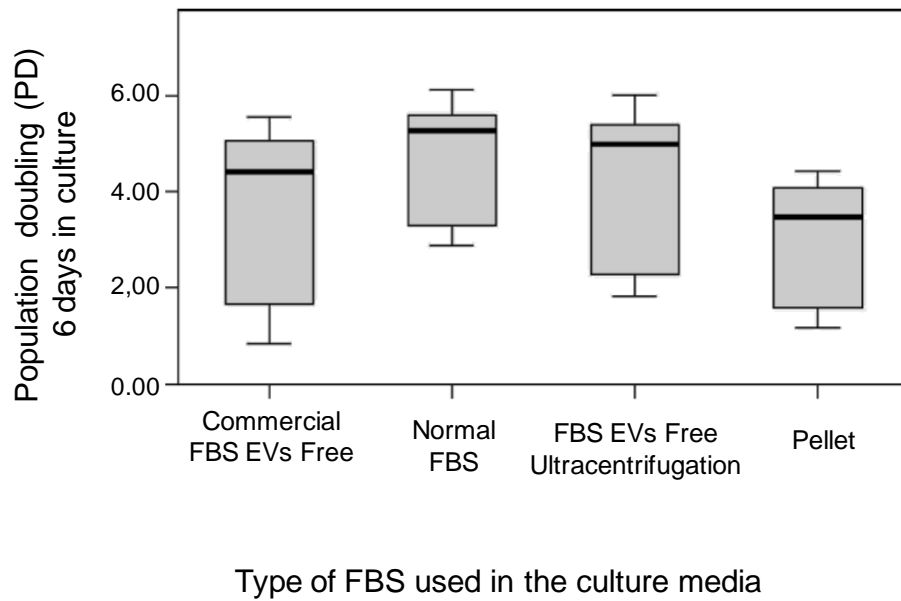
**Figure 22 Population doubling.** Graphs represent population doubling from different types of FBS used in BM cell cultures.

When comparing the morphology of commercial EVs depleted medium and the ultra-centrifuged exosome-depleted FBS with the control condition (DMEM supplemented with regular FBS), the cells showed a similar fusiform elongated morphology. When the medium was supplemented with the pellet resulting from the ultracentrifugation of regular FBS, MSCs morphology was different as cells appeared smaller and had a more elongated shape (**Figure 23**). Even though the growth rate didn't change, the difference in morphology could be explained by the reduced content of growth factors in this last condition tested.
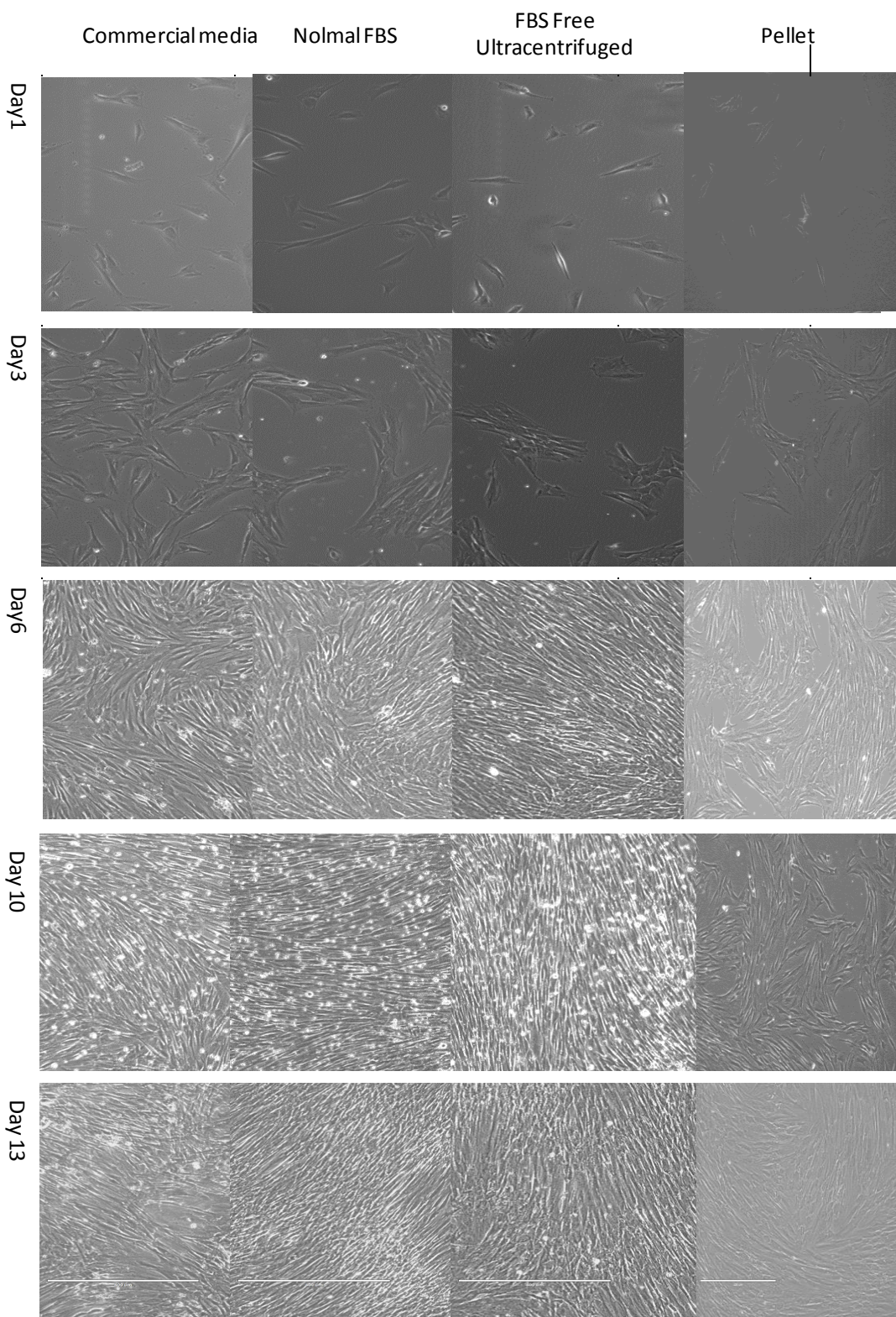
**Figure 23 BM-derived MSCs after being cultured with different media.** Representative images of BM-derived MSCs at different times in different culture conditions. Spindle like shaped cells appears similar in all type of conditions except when the pellet from the ultracentrifuge FBS was used. At day 13 in culture, all cells reached confluence in all the conditions (scale bar 250 µm).

*Isolation of EVs from different type of MSCs*

Collection of EVs from MSCs media was done after 24 hours in culture with EVs free FBS. Nanoparticle tracking analysis was done after the ultracentrifugation of media. This analysis allowed to observe the presence of particles having the expected reported size for EVs. We also analyzed the concentration of particles from each type of MSCs and the size distribution. **Table 6** shows the average results after the statistical analysis. Synovial fluid cells showed the highest amount of particles and bone marrow the lowest one, even though, after the statistical analysis, no significant differences ($p< 0.05$) were observed in their concentration.

**Table 6 Derived EVs from MSCs types**

| Cell type | Concentration of EVs (particles/ml) | Size (nm) |
|---|---|---|
| Bone marrow | $1,87 \pm 8,41 \times 10^{10}$ | $235,9 \pm 40,50$ |
| Synovial fluid | $1,69 \pm 2,47 \times 10^{11}$ | $180,2 \pm 64,45$ |
| Neck fat | $3,88 \pm 1,60 \times 10^{10}$ | $151,2 \pm 36,27$ |
| Mesenteric fat | $2,43 \pm 1,98 \times 10^{10}$ | $217,8 \pm 8,28$ |
| Tail fat | $5,85 \pm 4,36 \times 10^{10}$ | $153,8 \pm 62,24$ |

Data (n=3) is presented as average size ± SD

As mentioned before, **Table 6** represents EVs average results, but the Nanosight analysis software provides, for each analyzed sample, a histogram with the distribution of particles size. Size distribution histograms show that bone marrow cells produce an EVs population size ranging from 154 to 404 (**Figure 24 A**), while EVs produced by synovial fluid-derived MSCs range from 111 to 477 nm.
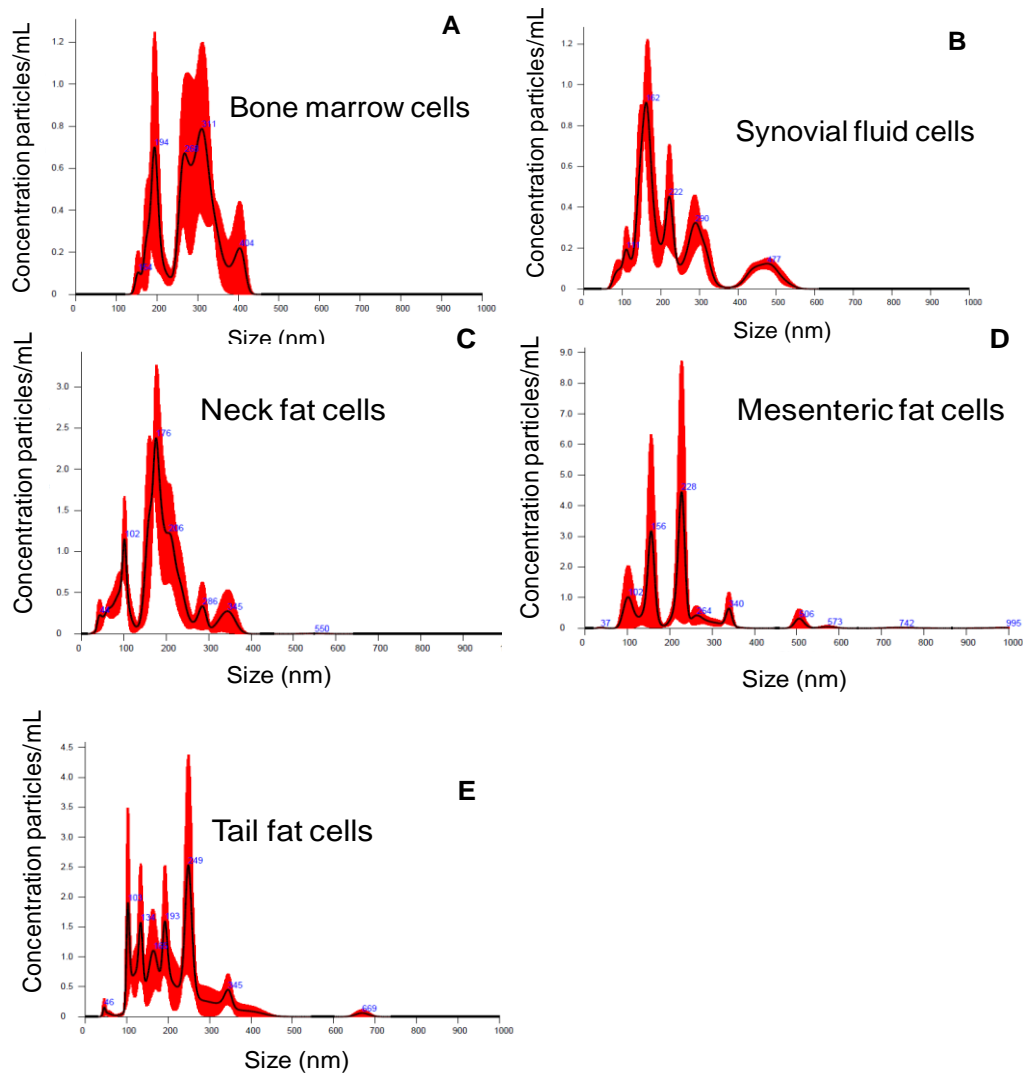
**Figure 24 Analysis of EVs size**. Histograms represent representative EVs size distribution calculated with the nanoparticle tracking analysis

EVs produced by adipose-derived MSCs seemed to be more heterogeneous in range. In the EVs from neck fat cells sizes from 40 to 550nm were observed. EVs from mesenteric fat-derived MSCs ranged from 37 to 742 nm, whereas tail fat-derived MSCs produced EVS from 46 to 669 nm.

*EVs morphology assessment with the transmission electron microscope (TEM)*

To confirm that the particles that were isolated from cultured MSCs were indeed EVs, isolated particle suspension were observed with a TEM. A magnification of 250k showed EVs with the described rounded "cup-shape" (**Figure 25**). We could observe EVs-derived from three types of MSCs (bone marrow, adipose and synovial fluid cells). EVs of different sizes were detected consistently with the nanoparticle tracking analysis.
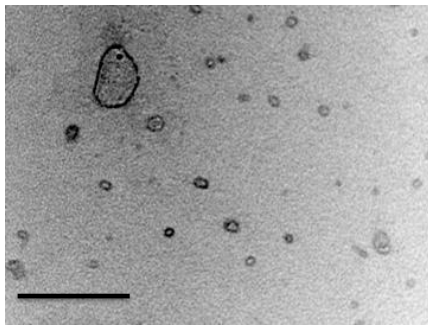
Bone marrow
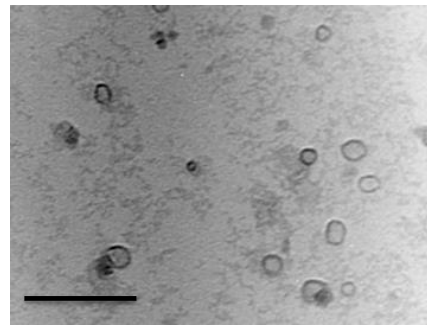


Neck fat
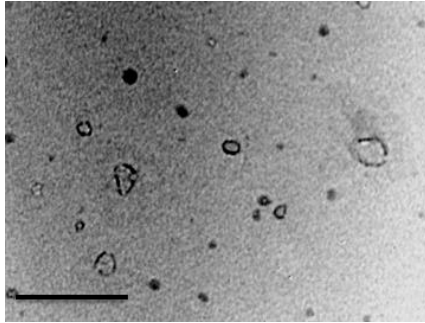


Synovial fluid

TEM Scale bar 0.125μm

**Figure 25 TEM of EVs-derived from MSCs.** Different types of sizes of EVs are observed with their rounded shape.

Immunostaining for CD9 helped to validate and confirm the identity of the EVs from the MSCs collected media. When stained for CD9, darker dots (**Figure 26**) could be observed on the surface of the EVs confirming the expression for such marker, as other authors also reported (Klymiuk et al., 2019).

Bone marrow



Neck fat



Synovial fluid

TEM Scale bar 0.05µm
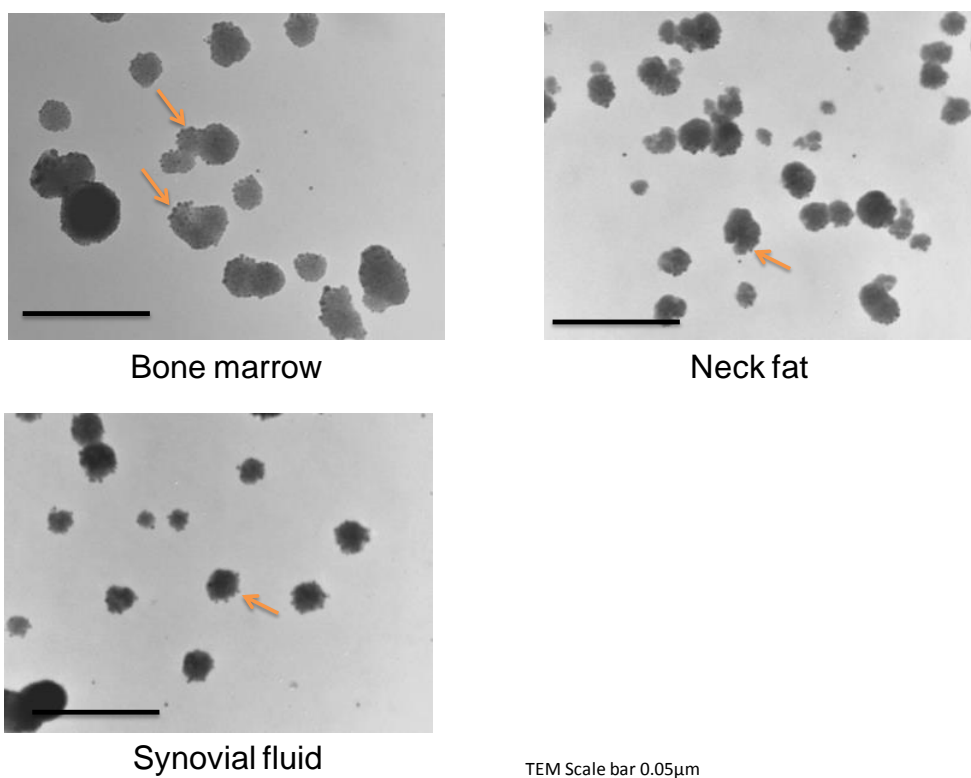
**Figure 26 CD9 labelling by immunogold.** Darker dots appearing as budding from the membrane represent CD9 positive staining (arrows).

## Aim 3: Evaluation of the effect of EVs in an inflammatory model

Independent assays were done using equine chondrocytes and treating them with EVs-derived from bone marrow, neck fat and synovial fluid cells and with each respective pro-inflammatory cytokine (IL-1β and TNFα). Such cytokines and the additional treatment with MSCs-derived EVs were added to evaluate whether they could counteract or reduce the effect of the pro-inflammatory cytokines on chondrocytes. For such treatments, cells needed to be starved in order to remove the effect of FBS on cells before the treatments. Cells were observed by the microscope to assure cell viability in such condition. After starvation, chondrocytes remained with their typical polymorphic morphology as shown in the control (**Figure 27**).

scale bar is 250 µm
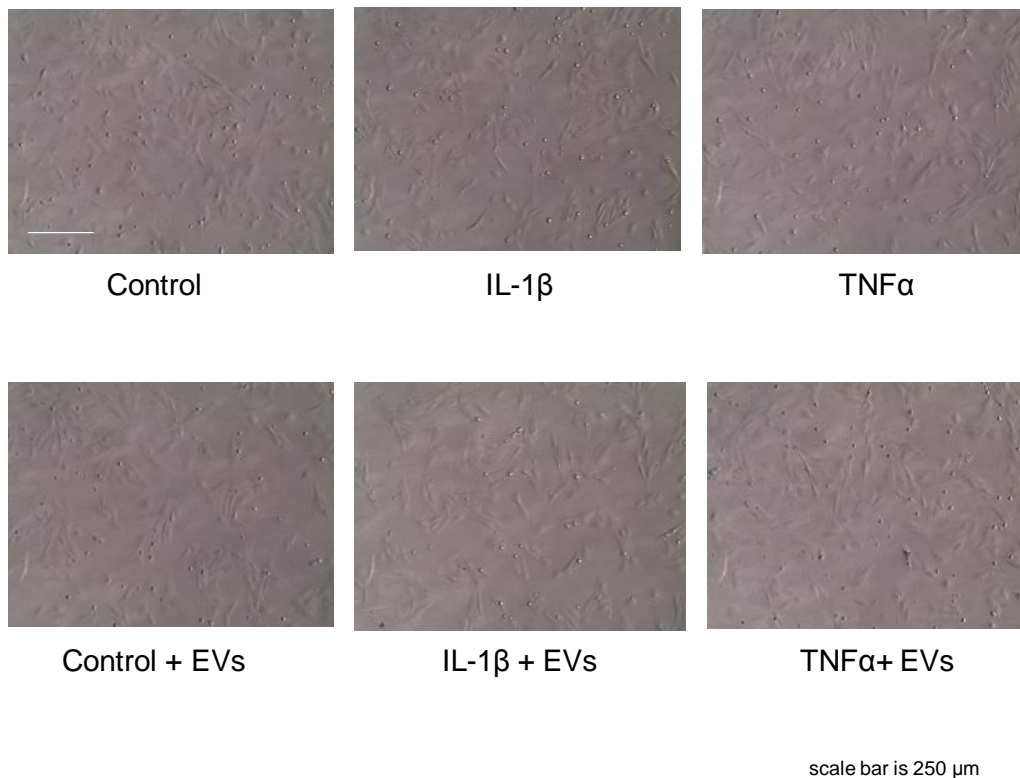
**Figure 27 Chondrocytes after 24 hours of treatment.** In the upper side, there are the treatments with cytokines and in the lower side, cytokines with the EVs. Images are representative of chondrocytes treated with BM cells-derived EVs.

Subsequently, chondrocytes were treated over 24 hours with either cytokines alone or either cytokines and EVs. Cell morphology among treatments was not different neither the approximate number of cells in each respective well as estimated visually.

Chondrocytes were analyzed by real-time PCR to evaluate gene expression. A list of genes (**Table 7**) evaluated and their main functions described in the literature are summarized as a guide to better follow this experiment.

**Table 7 Molecules involved during inflammation and OA described in literature**

| Gen | Function |
| --- | --- |
| IL-6 | Glycoprotein that enhances inflammation response |
| ADAMTS4 and ADAMTS5 | Enzyme that participates in the degradation of proteoglycans cartilage during OA (aggrecanase) |
| TIMP1 | Inhibitor of matrix metalloproteinases. Role in immunity and inflammation. |
| TIMP2 | Inhibitor of matrix metalloproteinases. Along with TIMP1 they seem to protect cells from apoptosis |
| TIMP3 | Inhibitor of matrix metalloproteinases. Stimulates cell proliferation |
| MMP1 | Matrix metalloproteinase (collagenase). Degradates collagen( I,II and III) |
| MMP2 | Matrix metalloproteinase (gelatinase) |
| MMP3 | Matrix metalloproteinase. Degradates collagen (II, III, IV, IX, and X), fibronectin, laminin and proteoglycans |
| MMP13 | Matrix metalloproteinase. Involved in the degradation of collagen (II, IV and IX), osteonectin and proteoglycan. Involved in normal remodelling of cartilage |
| SOX9 | Related with the chondrocyte phenotype. Important for chondrocyte cartilage production. |
| AGGRECAN | Structural in the extracellular matrix, a proteoglycan in cartilage |
| COLLAGEN II | Structure of the extracellular matrix, provides strength in cartilage |

*(Kidd et al., 2001; Schlueter and Orth, 2004; Verma and Dalal, 2011; Wojdasiewicz et al., 2014; Rose and Kooyman, 2016)*

*Bone marrow cells-derived EVs*

After chondrocytes were lysed to obtain RNA in order to analyze gene expression by real-time PCR, statistically significant differences were found in the IL-6 gene when comparing control (CTRL) with IL-1β treated cells and CTRL with IL-1β+EVs (p=.001) (**Figure 28**).
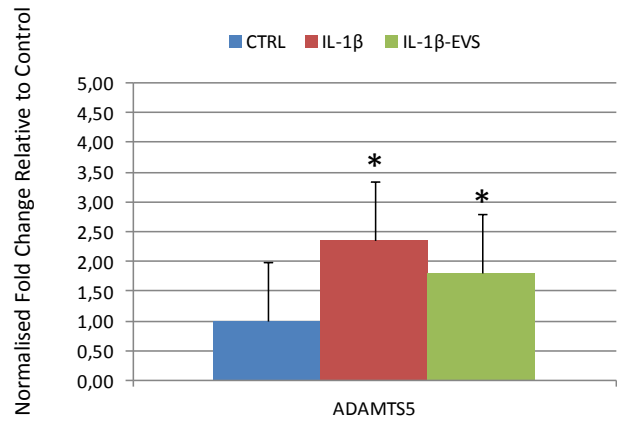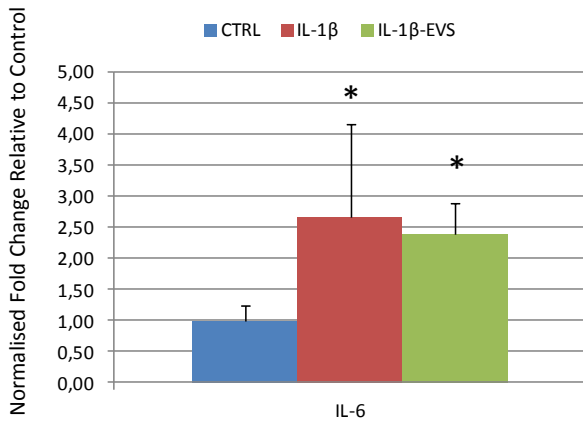
**Figure 28 IL-6 and ADAMTS5 genes after treatment with IL-1β and derived BM EVs.**
Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are represented by an asterisk (*) and were considered different at ($p<0.05$).

In the case of ADAMTS5 (**Figure 28**) when analyzing these results (CTRL) and IL-1β were significantly different ($p=.003$).
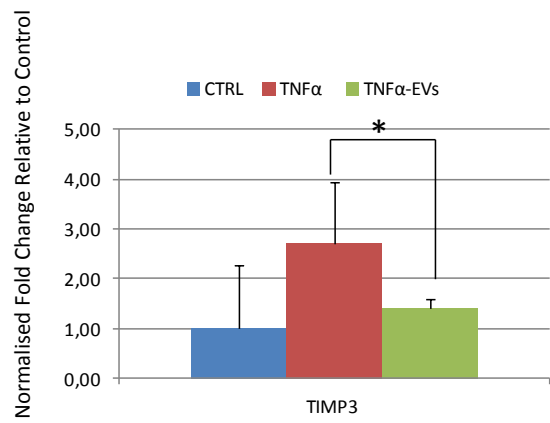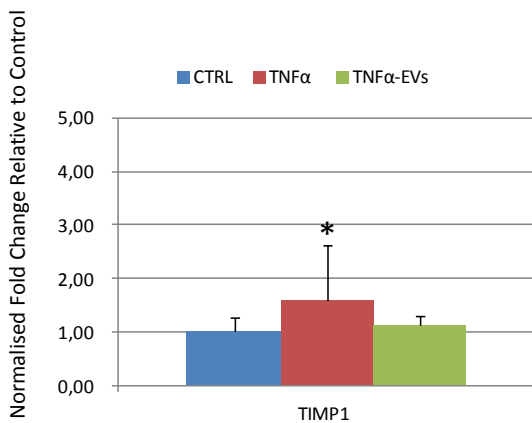


**Figure 29 TIMP1 and TIMP3 genes after treatment with TNFα cytokines and derived BM EVs.**
Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are represented by an asterisk (*) and were considered different at ($p<0.05$).

TIMPs expression was significantly different in the case of TIMP 1 and 3 (**Figure 29**). Significantly differences were observed in TIMP1 between CTRL and the TNFα ($p=.001$). In the case of TIMP3, differences were observed when comparing to TNFα and TNFα-EVs ($p=0.005$), we detected a tendency to decrease TIMP3 expression when chondrocytes were treated with EVs.
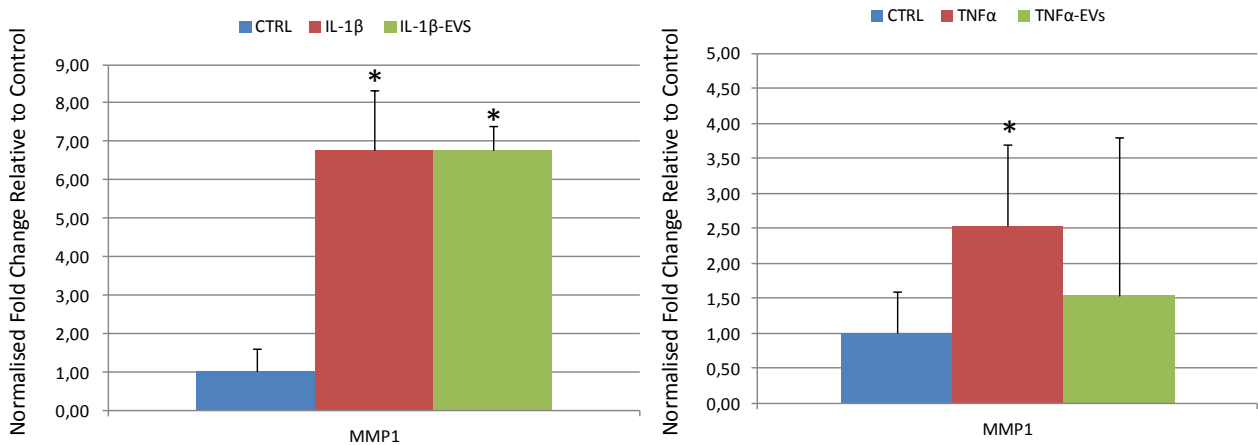
**Figure 30 MMP1 results after treatment with Il-1β and BM-derived EVs.** Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are represented by an asterisk (*) and were considered different at (p<0.05).

MMP1 gene showed significant differences when comparing CTRL with IL-1β and control with IL-1β–EVs (p<.001) (**Figure 30**). More interestingly, when analyzing the effect of TNFα, CTRL was statistically different when compared with TNFα treatment alone (p=.038), but no statistically significant difference was found when comparing CTRL with the co-treatment of TNFα and EVs.

**Figure 31 Metalloproteinase gene expression after treatment with IL-1β and derived BM EVs.**
Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours
of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are
represented by an asterisk (*) and were considered different at (p<0.05).

MMP2, 3 and 13 was significantly different in all the CTRLs against IL-1β, with a difference that
was particularly marked for MMP3 (fold change of 7, p<.001) (**Figure 31**). Whereas in MMP2
expression fold change was much lower (just above 2.18 fold-change) and MMP13 being in an
intermediate situation (**Figure 31**). Interestingly, a statistically significant decrease in MMP13
expression was found when chondrocytes were treated with the EVs along with IL-1β.

*Neck fat cells-derived EVs*

In the case of Neck fat (NF) MSCs-derived EVs, IL-6 expression seemed reduced only when co-
treatment of IL-1β and EVs was done. However, significant differences were observed against the
CTRLs for both cytokines (p<.001) when used alone (**Figure 32**).

**Figure 32 IL-6 and MMP1 genes expression after treatment with IL-1β and NF-derived EVs.** Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are represented by an asterisk (*) and were considered different at (p<0.05).

In MMP1 significant differences were observed between CTRL and IL-1β (p<.001) but such a difference disappeared when EVs were added as a co-treatment (**Figure 32**).
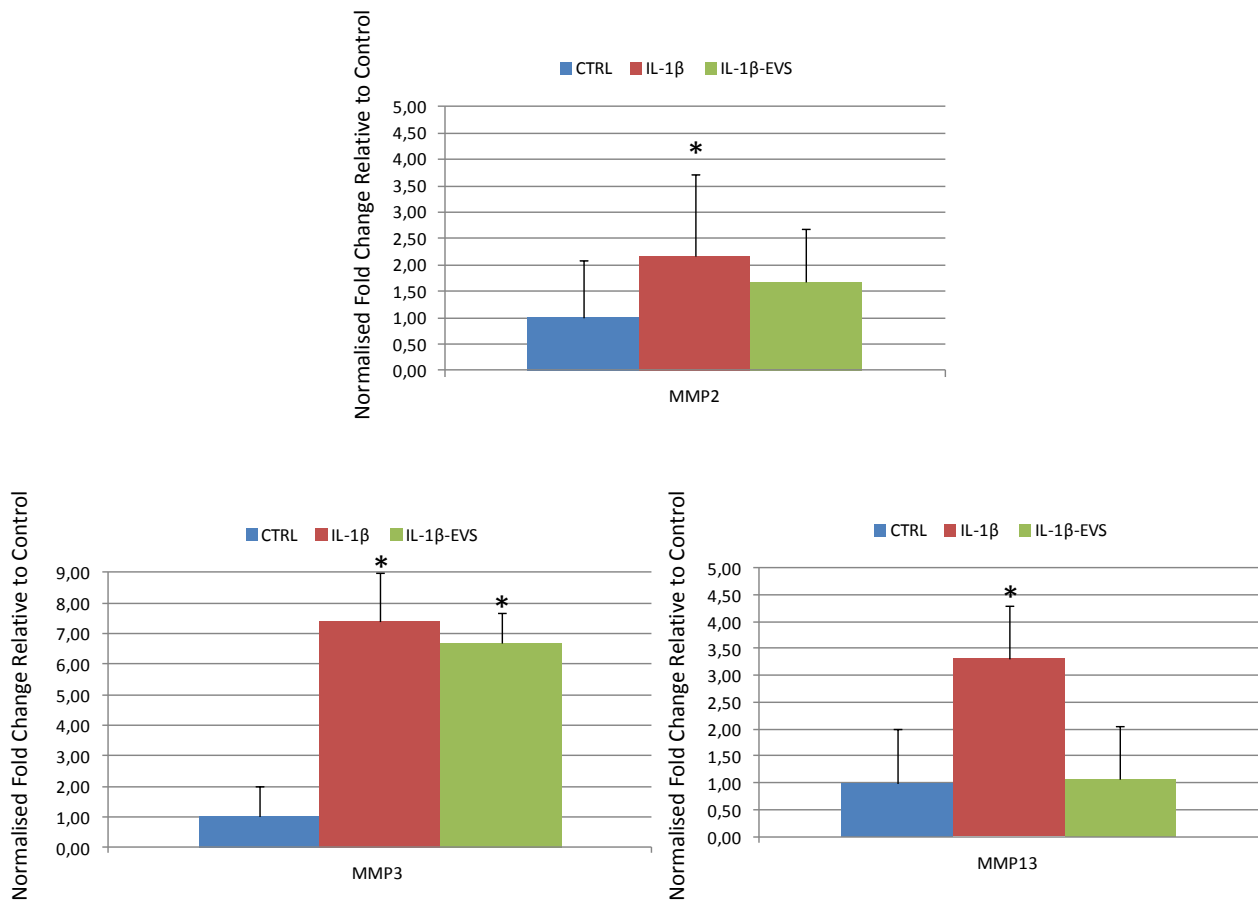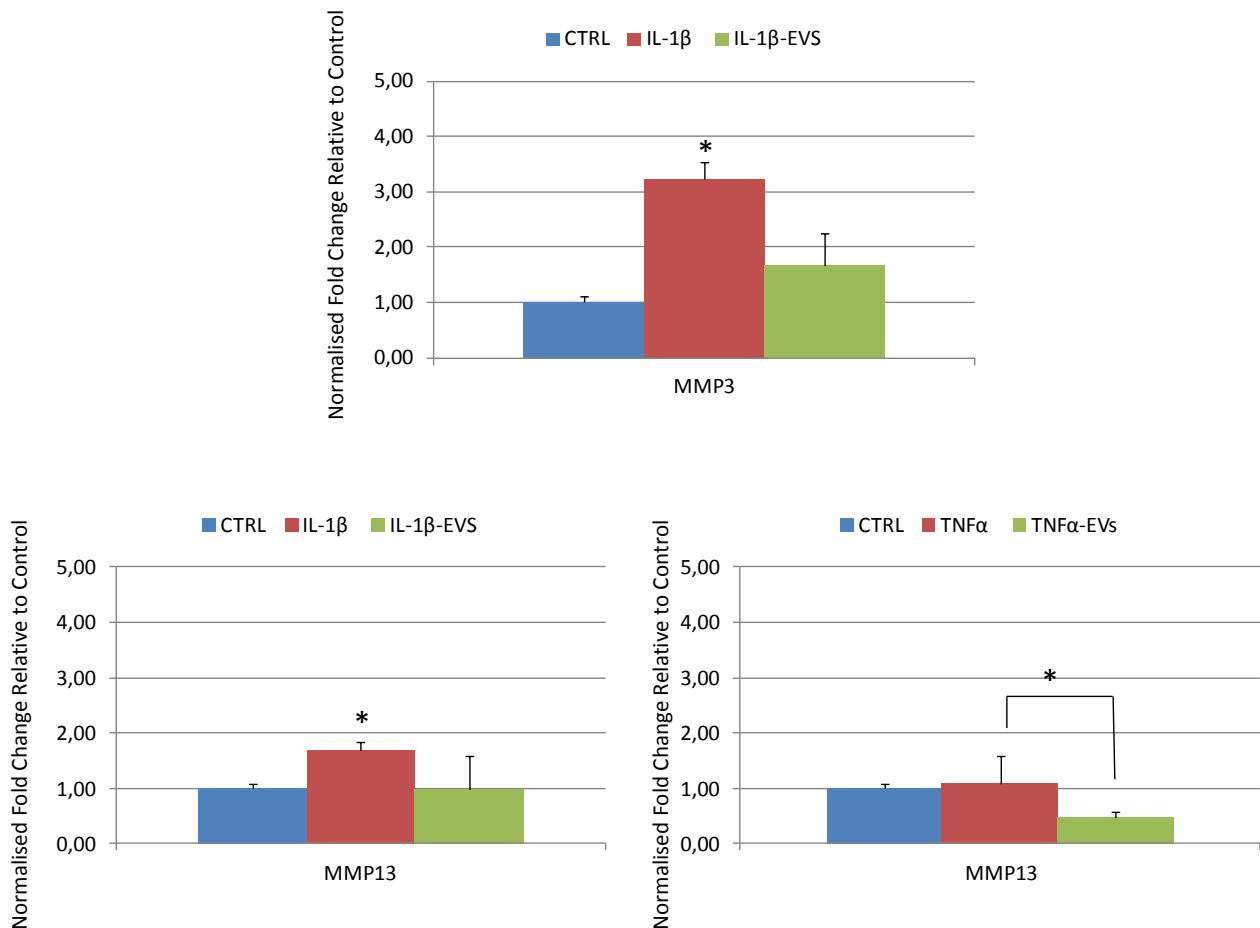
**Figure 33 Metalloproteinase gene expression after cytokines treatments and NF derived EVs.** Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are represented by an asterisk (*) and were considered different at (p<0.05).

The gene expression of MMP3 and MMP13 after adding EVs from NF MSCs was decreased (**Figure 33**). In the case of MMP3, significant differences were found in both cytokines against the CTRL (p=.004) (**Figure 33**) and differences against TNFα and TNFα-EVs were observed. (p=.008).

*Derived EVs from synovial fluid cells*

The effect of derived EVs from synovial fluid (SF) was also evaluated. However, significant differences were observed only comparing the IL-1β treatment against the CTRL for IL-6 (p=.014), but no differences were found between CTRL and co-treatment with IL-1β and EVs (**Figure 34**).
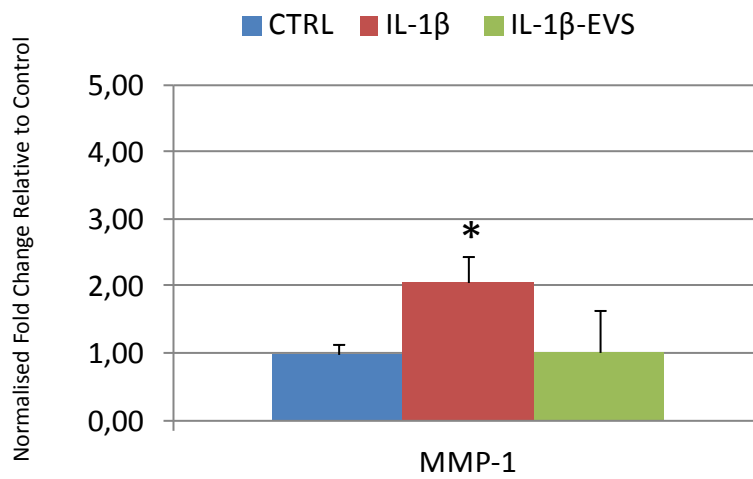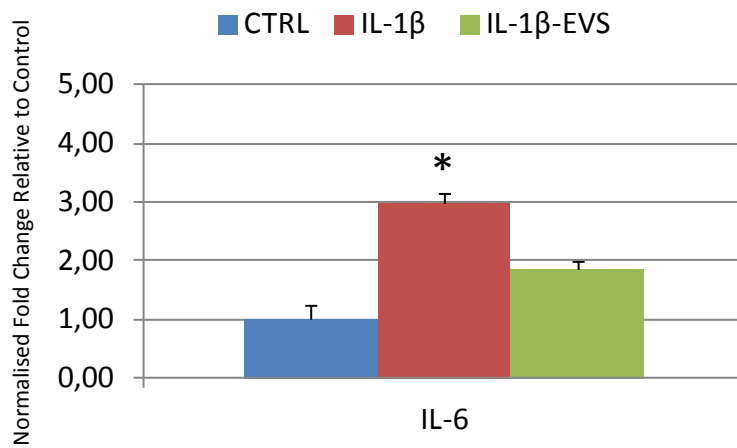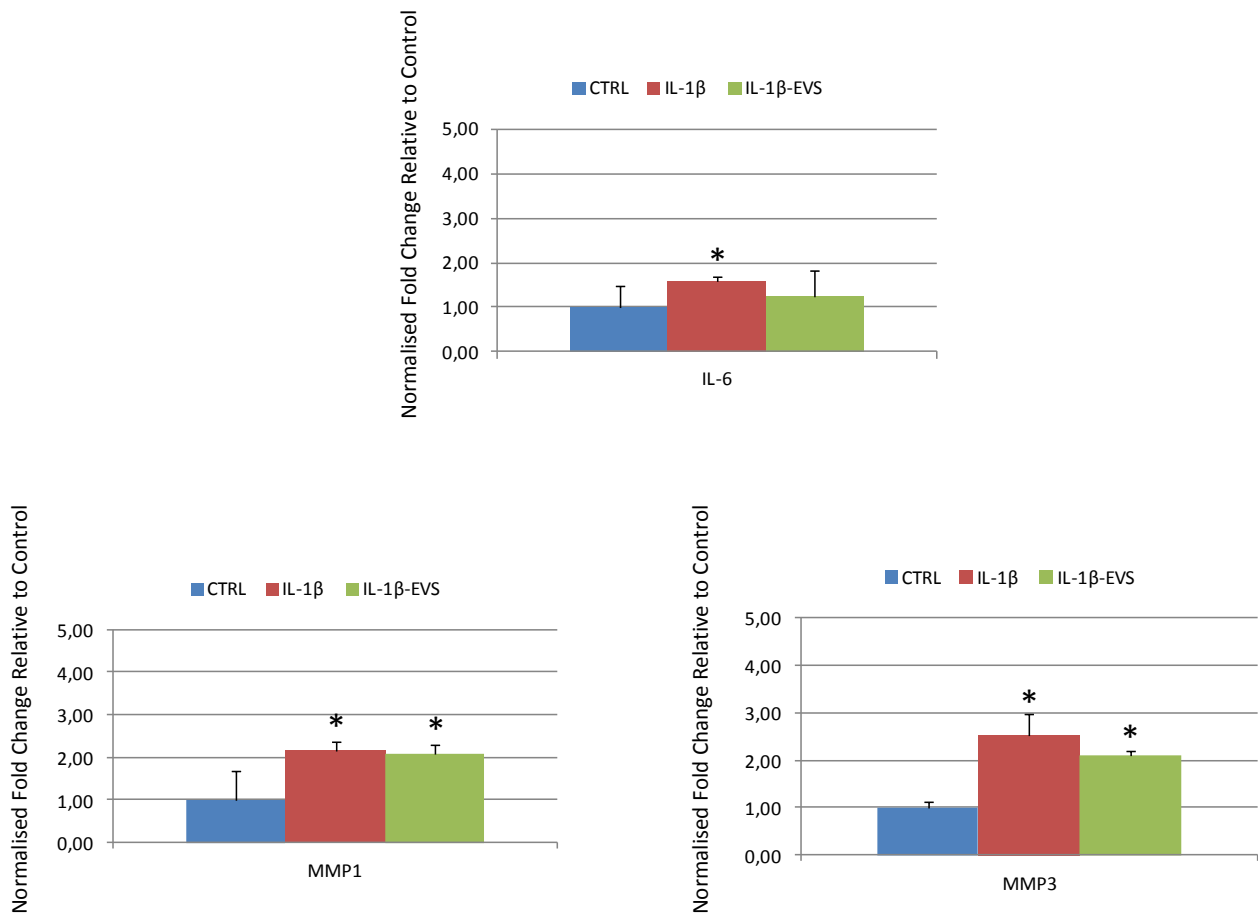
**Figure 34 IL-6, MMP1 and MMP3 gene expression after cytokines and SF-derived EVs.**
Histogram bars show the effect on chondrocytes relative gene expression to control after 24 hours
of EVs treatment. Bars represent the mean of ±SD from each group. Significant differences are
represented by an asterisk (*) and were considered different at (p<0.05).

The expression of MMP1 was significantly different in the case of CTRL against cytokine for IL-1β
and against CTRL versus IL-1β-EVs (p<.001). Statistically significant differences were observed
between the CTRL and the IL-1β and the CTRL against IL-1β –EVs (p<.001) also for MMP3
(**Figure 34**). No other comparisons for the rest of the analyzed gene were found to be statistically
different.

**Discussion**

**Aim 1: Isolation of MSCs from different tissue sources**

MSCs have been proposed and are now being used as therapeutic agents. The mechanisms through which they perform *in vivo* to act as treatments for several conditions are not well established, though. Since the overall aim of my PhD project was to gain a better insight into MSCs performance and mechanisms of actions, the first part of the PhD project focused on isolating MSCs from different tissue sources. MSCs are defined by specific characteristics that can, however, be influenced by their origin. Isolation from bone marrow, synovial fluid and adipose (neck, mesenteric and tail) tissues was performed. To ensure that proper MSCs were isolated, each of the criteria that have been defined and currently (Dominici et al., 2006b) accepted by the scientific community was tested:

1) Adherence to plastic was observed after tissue digestion and a fibroblast-like spindle-shaped morphology was observed.

2) The expression of specific surface antigens was analyzed and populations expressing CD90 and CD105 were detected in isolated cells, as other authors have reported (De Schauwer et al., 2011). A higher frequency of CD90$^+$ cells was found in the cells isolated from adipose tissues when compared with bone marrow and synovial fluid cells. Again, such a higher frequency was consistent with CD90 expression in adipose cells reported by other authors (Woeller et al., 2015). A significant frequency of CD105$^+$ cells was found in all analyzed tissues and higher again in the adipose tissue cells. The expression of such marker was previously thought to relate with the chondroprogenitor populations and potential in MSCs cells. However, in 2016, Cleary et al., (Cleary et al., 2016) showed that it should not be defined as a marker for such characteristics. It should be mentioned that chondrogenic potential might vary among donors when comparing the results here presented regarding the frequency of cells positive to such markers (Cleary et al., 2016). In contrast, our results showed that CD11α/CD18, CD45, CD79α and MHC II were very low or negatively expressed which is in accordance with other studies (Dominici et al., 2006; Murata et al., 2014). MHC II cell surface marker should not be expressed in cells that need to be implanted in horses. Most of the MSCs isolated in this present work had a frequency lower than 2% of MHC II$^+$ cells. However, approximately 3% of synovial fluid-derived cells were found to be positive for MHC II, hence exceeding the 2% threshold commonly accepted. It has been shown that MHC II expression might be variable (Schnabel et al., 2013). In our cells, this might relate with the immunologic state of joints at the moment of sampling at the slaughter. It has been demonstrated that the expression of MHC II reduces after passages (Schnabel et al., 2014). CD45$^+$ cells had also

a higher frequency (above 2% threshold) in the synovial fluid-derived cells. It was reported that this marker is mostly expressed in leucocytes (Schauwer et al., 2011), even though the presence of CD45^{+} cells in synovial fluid-derived cells does not relate with any ongoing pathologies.

3) Cell tri-lineage differentiation was evaluated. The cells were isolated from bone marrow, synovial and adipose tissues were able to go through differentiation towards a chondrocyte, osteocyte and adipocyte phenotype, as confirmed by the positive stainings for adipogenesis (Oil red O), osteogenesis (Alkaline phosphatase and Alizarin red) and chondrogenesis (Safranin red) as reported in literature (Schauwer et al., 2011).

After such results, we were able to confirm that the isolated cells from all of the examined tissues were indeed MSCs.

As the following step certain *in vitro* characteristics of these cells were evaluated and compared across the different sources. *In vitro* evaluation of MSCs by cell growth, migration and wound healing test allowed to better understand MSCs performances. Cell growth analysis after 24 hours and 48 hours demonstrated that synovial fluid possess a faster growth potential than the other isolated cell types. Such a characteristic might be important for cell expansion *in vitro* before reimplantation in order to reduce the time occurring between isolation and reimplantation. Usually, after isolation of MSCs, the *in vitro* expansion can take up to weeks before being implantation can be carried out (Smith, 2008). The migration study allowed to compare the migration capacity of all the derived MSCs. It could be observed that BM-derived cells were the ones that had a higher migration capacity when compared with other types of cells. After reimplantation, migration to the wound area and persistence of inoculated cells are important in determining the outcome of the treatments (Smith et al., 2012). A higher *in vitro* migration capacity might relate to such *in vivo* properties and therefore provide a simpler evaluation for the selection of the most suitable tissue source to use for isolating MSCs. The wound healing test takes into account and evaluates concomitant processes of proliferation and migration on a bi-dimensional surface to fill an artificially made gap. This characteristic showed that using conditioned medium from BM-derived cells improved the filling of the gap showing a better capacity to influence and modulate cell movement and migration of other cell types as well (Cabezas et al., 2018). After such results, we decided to focus on the paracrine factors that might be involved in exerting this capacity and as such, we decided to focus more specifically on extracellular vesicles secreted by MSCs.

**Aim 2: Isolation of EVs from different types of MSCs**

To study EVs the use of appropriate media and FBS for culturing MSCs is important in order to obtain pure EVs, reducing contamination and diminishing misinterpretation in results (Wei et al., 2016). We, therefore, decided to use the ultracentrifugation as a methodology to eliminate EVs from FBS before adding it to the MSCs cultures. Our results coincided with those reported by the

use of ultracentrifuged FBS showing that cells were able to grow and retained cell viability (Schwartz, n.d.) reducing the presence of other EVs and contaminants. EVs removed from FBS were from bovine serum and their properties were not assessed since these contaminants in the FBS were in the assay set-up, not a variable to study. In addition to this, we could observe that MSCs morphology was not modified. This helped and was beneficial for later studies in which a variable (the use of a completely different FBS-free medium) could be eliminated and MSCs could be grown for EV isolation in a medium almost identical to their own proliferation medium. As culture medium might influence the secretion of EVs, such a change could have had an undesired effect on EVs characterization.

The isolation of EVs from different type of MSCs showed that the concentration of EVs from different type of MSCs was not different. The concentration of adipose EVs was marginally higher than what has been reported for equine cells in the literature (Klymiuk et al., 2019). For example, when considering adipose tissue obtained from similar regions (tail base), $5.85 \times 10^{10}$ particles/mL were obtained in the present study while Klymiuk et al. obtained $9.6 \times 10^{8}$ particles per mL. Evidently, differences between cell culture conditions might bring to variations depending on the size of cell dish and length of time before media collection. In respect to EVs' size, no significant differences were found among the different types of MSCs studied here. BM-derived EVs were approximately 235nm. EVs-derived from human BM MSCs have shown that they could reach sizes up to 151-241 nm (Tracy et al., 2019b). Adipose-derived EVs from equine in our study showed that the bigger ones were approximately 151 and 217 nm. The literature on equines has reported size of 353 nm for EVs from adipose cells (Klymiuk et al., 2019). In both cases, the size of EVs from human and the few reports that exist from EVs from equines differ due to the type of methodological protocol for isolation. That is, differences in EVs' size have been observed in the same type of cells after using different isolation protocols (ultrafiltration, ultracentrifugation, precipitation and size exclusion chromatography) (Klymiuk et al., 2019). When analyzed with the TEM, EVs showed CD9 expression as round spheres could be detected with darker dots after performing the immunogold staining similar to what has been reported by another study (Klymiuk et al., 2019).

**Aim 3: Evaluation of the effect of EVs in an inflammatory model**

In the present study, equines chondrocytes were used as a model to evaluate the effect of pro-inflammatory cytokines (IL-1β and TNFα) which are related to osteoarthritis (Wojdasiewicz et al., 2014). In most of the treatments, adding IL-1β to chondrocytes resulted in an increase in the expression of IL-6. The importance of interleukin-6 is because of its role during OA where it activates the immune system and produces an inflammatory response by the presence of cytokines IL-1β and TNFα. (Wojdasiewicz et al., 2014). Our results coincide with other studies in

equine chondrocytes where it has been observed an up-regulation of the IL-6 gene after treatment with IL-1β (David et al., 2007). It has been suggested that there is a relationship between IL-1β and IL-6 expression in chondrocytes, meaning that when there is the presence of IL-1β there is the production of IL-6 in exposed tissues (Fan et al., 2007; Wojdasiewicz et al., 2014). When EVs-derived from BM, synovial and adipose cells in chondrocytes treatments were added as co-treatments, in some of them a tendency to reduce IL-6 expression was observed. However, in all of the cases, such differences were not statistically significant. A reduction of IL-6 has been reported in stimulated chondrocytes with cytokines when treated with derived BM EVs *in vitro* (Vonk et al., 2018). Other molecules, like transcription factors or enzymes such as COX2 that are involved in the inflammatory pathway (Vonk et al., 2018) could have been evaluated. The expression of ADAMTS5 in our study was increased significantly as expected in IL-1β treatments. This ADAMTS5 along with ADAMTS4 are known to be present during cartilage degradation in OA (Verma and Dalal, 2011). We could confirm that cytokine IL-1β induced the expression of this aggrecanase and that when adding EVs-derived from BM, a reduction pattern was observed but again it did not reach statistical significance. It has been reported that ADAMTS4 seems mainly stimulated by cytokines when compared with another type of aggrecanases (Wojdasiewicz et al., 2014), however, in our study, we did not found significant differences in its expression. TIMPs are important modulators of ECM remodeling and the expression of TIMPs after cytokine effect seems to be generally reduced. For example, during OA, TIMP3 seems to be downregulated (Kidd et al., 2001). However, such a statement is not completely true since TIMP3 balance with metalloproteinase expression is not just related to pathological OA. The remodeling of the extracellular matrix is also present in healthy patients. Even more, TIMP3 increase in cells has been also related to stimulation of cell growth and proliferation (Lambert et al., 2004). In the present study, the expression of TIMP 1 and 2 in chondrocytes treated with TNFα increased. When considering TIMP3 expression in TNFα treated chondrocytes against TNFα + BM-derived EVs there were significant differences: TNFα treatment resulted in an increase in TIMP3 expression which reverted to control levels when EVs were added. However, further studies to better understand TIMP expression changes in equines and EVs effect on such genes should be performed. MMPs should be up-regulated when during inflammation (and therefore exposure to IL-1β and TNFα). IL-1β treated chondrocytes showed a significant increase of MMP1 expression against the control, however no differences were observed when adding BM-derived EVs. However, when adding EVs- derived from NF, the MMP1 expression was reduced. Interestingly, this decrease results in expression levels comparable to those found in the control samples. The expression of MMP1 is usually higher during OA (Zeng et al., 2015), therefore the effect of MMP1 in derived NF EVs shows a promising result. In the case of MMP1 in treated TNFα chondrocytes, the effect was not as high as when they were treated with IL-1β. For MMP3 and MMP13, their expression was increased during cytokine treatments similarly to other studies (Wojdasiewicz et al., 2014; Linardi et al., 2019). Significant differences were observed in MMP3 expression after the

addition of NF- derived EVs: a decrease was evident when compared to IL-1β treated cells even though MMP3 expression was still higher than the control samples. When adding BM-derived EVs, a significant reduction of MMP13 was observed similar to what was detected when NF-derived EVs were added along with IL-1β. These valuable results from NF and BM-derived EVs showed that they might affect the regulation of the extracellular matrix remodeling during inflammation.

The results obtained from this PhD project demonstrate that MSCs can be isolated from different types of tissues (bone marrow, synovial and adipose tissues). However, synovial fluid cells grow faster than other MSCs types and they can be expanded faster *in vitro* after isolation. Bone marrow cells showed a better migration and a better effect on non-mesenchymal cell types as evident in the wound healing assay. These *in vitro* results might be useful to select the harvesting site for MSCs isolation and  the evaluation of their *in vitro* characteristics might be important for  the decision making process for *in vivo* treatment of equine leg pathologies. However, some MSCs harvesting sites like synovial fluid, mesenteric and neck fat cells should be more extensively studied for ensuring their viability for *in vivo* use.

Overnight high-speed ultracentrifugation of FBS is a practical and economical methodology for the culture of MSCs for EVs isolation purposes. This allows growing MSCs without modifying *in vitro* culture conditions and reducing adaptation periods for different types of media. Different MSCs types derived EVs were evaluated for the first time in this report. The amount and size of EVs-derived from bone marrow, synovial and adipose tissue are similar. However, differences were observed between their effects on gene expression during inflammation. BM-derived and NF-derived EVs seem to have a promising effect mainly on MMPs (1, 3 and 13) expression. However, further studies should be performed by increasing the amount of EVs or co-incubation time. Moreover, the analysis of the content of EVs (RNAs more specifically or selected growth factors) might help to understand their effect. To date, little is known about EVs content and about their effectiveness as a treatment to improve inflammation in equines so the present study provides a novel approach with *in vitro* data that might help adapt a more aware approach to the *in vivo* use of MSCs or of their derived products in equine leg pathologies.

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