UNIVERSITÀ DI PARMA

Dottorato di Ricerca in Scienze Medico-Veterinarie

Ciclo XXX

The CXCR4/SDF-1 α axis: target for the migration enhancement of canine adipose-derived mesenchymal stem cells

Coordinatore:

Chiar.mo Prof. Attilio Corradi

Tutor:

Chiar.mo Prof. Antonio Cacchioli

Co-tutor:

Chiar.mo Prof. Stefano Grolli

Dottorando: Dott.ssa Ana Ivanovska

Anni 2014/2017

Index

Abstract

Mesenchymal stem cells are multipotent adult stem cells, capable of self-renewal, differentiation into other cell types, production of bioactive factors and migration and homing to inflammation and injury sites. In veterinary medicine, MSCs are currently considered a promising therapeutic tool and have been used for the treatment of different clinical conditions.

The goal of the present PhD thesis was to study the biological and clinically relevant features of canine MSCs, and to optimize cell culturing protocols for improved therapeutic effects. In the first part, we characterized canine adipose derived mesenchymal stem cells from perivisceral and subcutaneous adipose tissue, based on their immunophenotype with species-specific canine antibodies. In the subsequent study, we evaluated the molecular mechanisms of MSCs migration towards different chemiotactic gradients, simulating the *in vivo* environment. Finally, we proposed an *in vitro* modulation system aimed to increase cell proliferation, and gene and protein expression of molecules involved in MSCs migration and the homing to the target site, with the final aim of increasing their therapeutic efficacy.

Canine adipose-derived mesenchymal stem cells deriving from perivisceral and subcutaneous adipose tissue showed a similar immunophenotypic profile. The CXCR4/SDF-1α axis was responsible for cell migration. However *in vitro* passages reduced the expression of CXCR4 receptor, which can hamper the ability of the cells to migrate to the lesion site. The synergistic effect of hypoxic pre-conditioning and bFGF increased the gene expression of CXCR4, and cytoplasmic expression of the protein CXCR4. Additionally, hypoxic pre-conditioning increased the SDF-1 gene expression. However the surface protein expression remained negative, indicating that additional stimuli are needed for the increase of CXCR4 on the cells.

The present study reports for the first time the characterization of canine adipose-derived mesenchymal stem cells with a panel of species-specific antibodies that can be easily applied for accurate cell characterization in pre-clinical studies and in the clinical setting. The expression of CXCR4 receptor and its ligand SDF-1 in canine adipose-derived mesenchymal stem cells were studied for the first time in consecutive passages (P0-P3) and were found to be responsible for cell migration. Finally, for the first time the synergistic effect of hypoxia and bFGF on mesenchymal stem cells was studied and the promising results indicate that it can be used for cell preconditioning.

Riassunto

Le cellule staminali mesenchimali (MSC) sono delle cellule staminali adulte capaci di auto-rinnovarsi, di differenziarsi in altri tipi cellulari, di produrre fattori bioattivi e di migrare verso il sito della lesione. Nella medicina veterinaria, le cellule staminali mesenchimali sono considerate come un agente terapeutico promettente.

Lo scopo della presente tesi di dottorato, è stato quello di studiare le caratteristiche biologiche delle MSC derivate da tessuto adiposo di cane, per contribuire da un punto di vista biologico a una adeguata caratterizzazione e ottimizzazione dei protocolli di coltura cellulare, con il fine di ottenere una maggiore efficacia terapeutica nella clinica veterinaria. Nella prima parte del lavoro di tesi, è stato caratterizzato l'immunofenotipo delle MSC di cane isolate da tessuto adiposo periviscerale e sottocutaneo, utilizzando un panel di anticorpi specie-specifici. Nella parte successiva del lavoro, sono state valutate, in vitro, le basi molecolari della migrazione di MSC di cane, simulando l'ambiente *in vivo*. Infine, è stato proposto un modello di coltivazione in vitro di queste cellule per aumentarne la proliferazione cellulare, e l'espressione dei geni e delle proteine coinvolte nella migrazione delle cellule, con lo scopo finale di aumentare la loro efficacia terapeutica.

Le cellule derivanti dal tessuto adiposo periviscerale e sottocutaneo sono risultate simili in base al loro profilo immunofenotipico. Il panel di anticorpi utilizzato nella analisi ha permesso di ottenere un quadro di caratterizzazione che rispecchia quanto atteso in base alla letteratura scientifica, per la prima volta basato esclusivamente su anticorpi specifici per antigeni del cane. L'asse CXCR4/SDF-1 α è responsabile della migrazione delle MSC. I passaggi in vitro, come osservato in altre specie, hanno ridotto l'espressione genica e proteica di CXCR4, fatto che

potrebbe influenzare negativamente la migrazione delle cellule verso il sito della lesione. L'effetto sinergico del pre-condizionamento delle cellule ottenuto mediante coltura in ambiente ipossico ed in presenza di bFGF ha aumentato sia l'espressione del gene CXCR4 che l'espressione citoplasmatica della proteina CXCR4. Inoltre, il pre-condizionamento ipossico ha aumentato l'espressione del gene SDF-1. Entrambi questi dati indicano una attivazione dell'asse CXCR4/SDF-1 α in queste condizioni, anche se, in ogni caso, la localizzazione sulla superficie cellulare della proteina CXCR4 è rimasta negativa.

La presente tesi riporta quindi per la prima volta la caratterizzazione di MSC di cane da tessuto adiposo, con un set di anticorpi specie-specifici che può essere prontamente usato in studi pre-clinici e nella clinica. L'espressione del recettore CXCR4 e del suo ligando SDF-1α è stata studiata per la prima volta in cellule staminali mesenchimali di cane, durante passaggi consecutivi *in vitro* (P0 – P3) confermando il ruolo di questa via di segnalazione tra cellule come responsabile della migrazione di MSC del cane. Infine, per la prima volta è stato studiato l'effetto sinergico dell'ipossia e del bFGF su MSC, ottenendo risultati promettenti al fine dell'ottimizzazione delle colture di MSC, non solo nel cane, ma anche in altre specie, compreso l'uomo, al fine di ottenere una loro migliore efficacia terapeutica.

Chapter 1: Introduction

1. 1. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are adult multipotent cells of mesodermal derivation, located in the perivascular niche in various tissues and post-natal organs. They are fibroblast-like self-renewable cells which are easily expandable *in vitro*, characterized by genomic stability and low impact ethical concerns which overall, made them useful for clinical applications in cell-based therapies, regenerative medicine and tissue regeneration models (Rohban and Pieber, 2017). MSCs have been firstly isolated from bone marrow (Friedenstein et al., 1970). Cells with similar characteristics have been isolated from adipose tissue (Bunnell et al., 2008), amniotic fluid (Moraghebi et al., 2017), amniotic membrane (Manuelpillai et al., 2011), dental tissues (Huang et al., 2009; Seifrtova et al.,2012), endometrium (Schuring et al., 2011), limb bud (Jiao et al., 2012), menstrual blood (Allickson and Xiang, 2011), peripheral blood (Ab Kadir et al., 2012), placenta and fetal membrane (Raynaud et al., 2012), salivary gland (Rotter et al., 2008), skin and foreskin (Bartsch et al.,2005, Riekstina et al., 2008), sub-amnioic umbilical cord lining membrane (Kita et al., 2010), synovial fluid (Morito et al., 2008), and Wharton's jelly (Wang et al., 2004; Hou et al., 2009). Resident MSCs present in the organism have been poorly characterized, however numerous studies have been conducted on MSCs isolated and expanded *in vitro*. In order to standardize the populations of MSCs, The International Society for Cellular Therapy has proposed three minimal criteria that define them: a) adherence and proliferation on plastic surface; b) *in vitro* differentiation into adipogenic, osteogenic and chondrogenic lineage and c) positive expression of cells surface markers CD73, CD90 and CD105 (≥95% positive

cells) and negative expression of CD14 or CD11b, CD34, CD45, CD19 or CD79α, and MHC-II. (≤2% negative cells) (Dominici et al., 2005).

1.2. Therapeutic mechanisms of MSCs

The therapeutic potential of mesenchymal stem cells has been acknowledged, however the precise mechanism of action is still unknown. Based on current findings, four significant key mechanisms of action are responsible for their beneficial effect: plasticity and differentiation in multiple cell types, immunomodulation, secretion of bioactive factors and homing (Fig.1). In case of specific diseases and conditions, multiple mechanisms of action contribute to the therapeutic effect of MSCs (Wei et al., 2013).

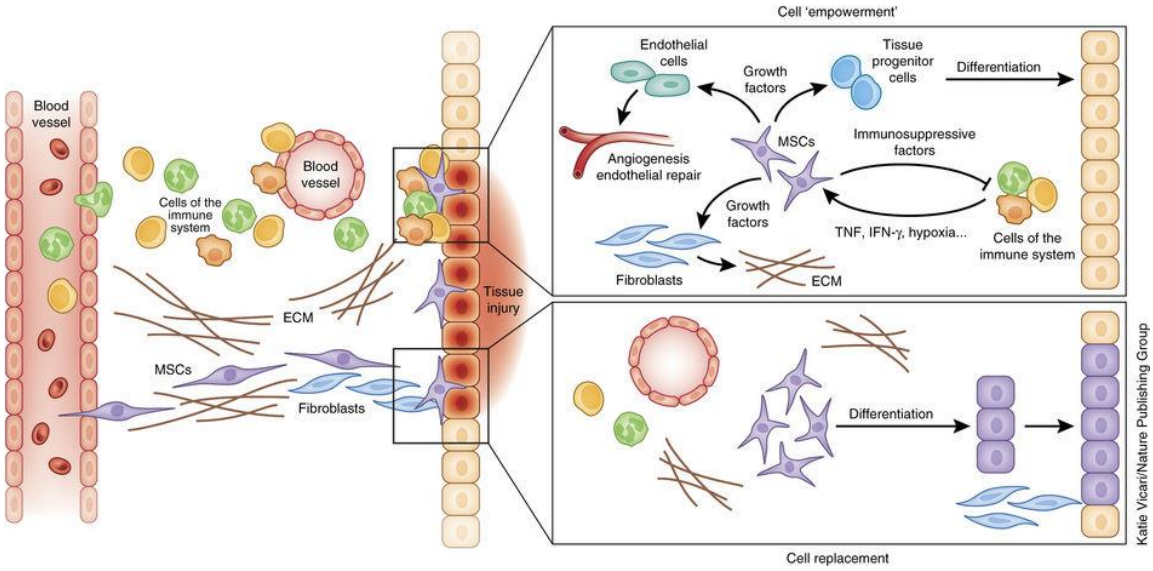

Fig 1: Mechanisms of action of mesenchymal stem cells *(Wang et al., 2014)*

1.2.1. MSCs plasticity and self-renewal

Stem cells are responsible for the maintenance of tissue and organ homeostasis. In case of injuries, local resident stem cells contribute to tissue repair by the ability of self-renewal and differentiation in organ-specific cells. Indeed, one of the three defining criteria for MSCs *in vitro* is the tri-lineage differentiation in adipogenic, chondrogenic and osteogenic lineage. *In vitro* protocols have been standardized with known and limited growth factors and nutrients for their differentiation. However, there is evidence that stem cells can differentiate in cells not originally present in the native tissue. This plasticity makes them able to transdifferentiate and contributes to the regeneration of tissues deriving from the same or different germ layer, resulting in intra-germ layer conversion or inter-germ layer conversion (Eisenberg et al., 2003; Raff, 2003; Rutenberg et al., 2004; Quesenberry et al., 2004) (Fig. 2). For example, under defined conditions neural stem cells can give origin to hematopoietic (Bjorsnon et al., 1999) or myogenous cell (Galli et al., 2000); bone marrow mesenchymal stem cells to neural and glial cell (Kopen et al., 1999), cardiomyocytes (Orlic et al., 2001), pneumocytes (Krause et al., 2001) and hepatocytes (Lagasse et al., 2000). The transdifferentiation protocols are extremely complex since the conditions need to be precisely defined and a numerous specific pre-conditioned growth factors need to be present (Gregory et al., 2005). Another aspect to take in consideration is the fact that MSCs populations are heterogeneous and different clones are present within it, so even with an appropriate medium the desirable differentiation effect is not obtained for every single cell (Baksh et al., 2004; Pevsner-Fischer et al., 2011; Ylostalo et al., 2008). An important condition to take in consideration is the additive effect of the cell seeding density and number of passages,

especially for the chondrogenic differentiation. The chondrogenic cascade is coordinated by surface molecules; therefore the proximity of the cells in three-dimensional systems leads to the formation of micromasses (Tuli et al., 2003, Gregory et al., 2005).

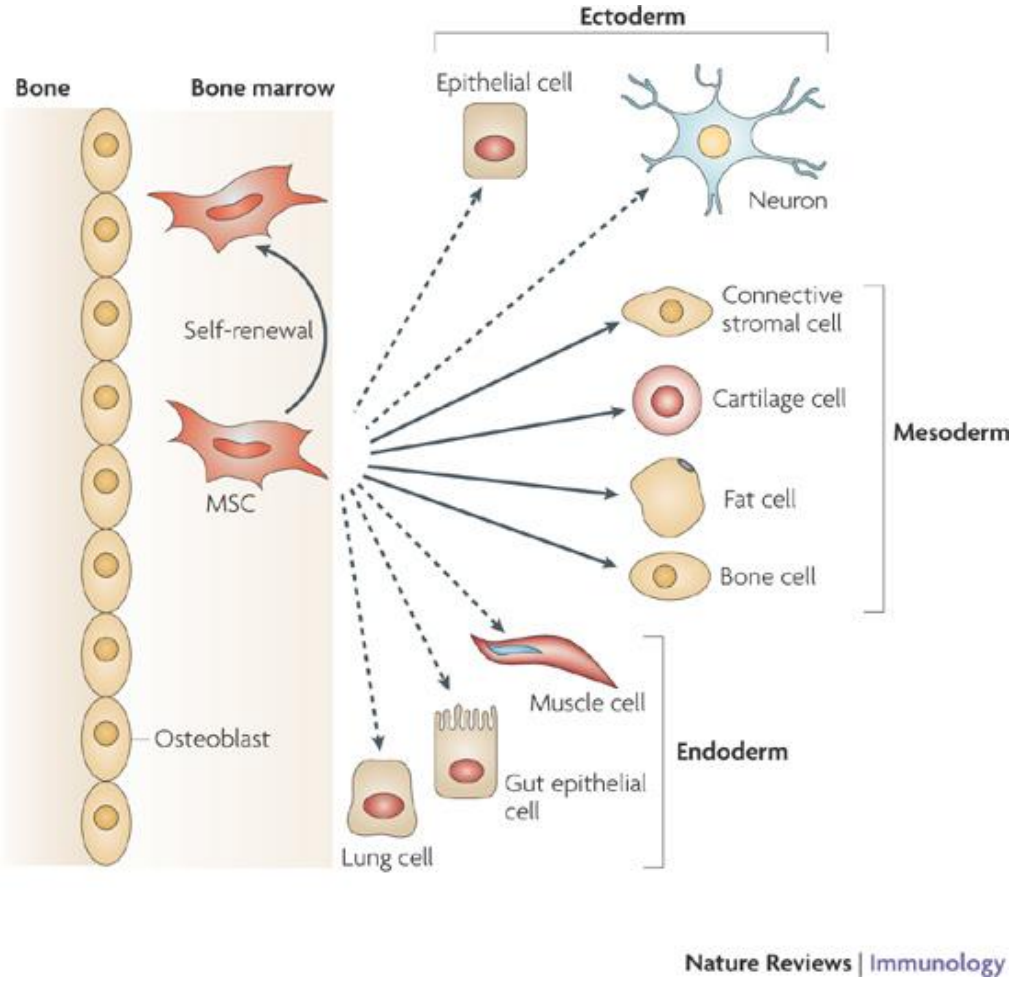

Fig 2: Multipotency of MSCs *(Uccelli et al., 2008)*

From the clinical point of view the differentiation properties including the possibility of *in vitro* expansion, makes MSCs suitable for tissue repair and gene therapy (Gregory et al., 2005). For a successful clinical application, MSCs need to be expanded *in vitro* for several passages to obtain a sufficiently high number of cells readily useful in the clinical setting. The cell density influences the *in vitro* expansion of MSCs. Lower cell seeding densities promote high expansion potential and on the contrary high densities lead to low cell expansion (Colter et al., 2000). However long-term *in vitro* expansion causes cell modifications and development of genomic alterations, which lead to cell senescence that alters the therapeutic potential of MSCs. In particular, cells loose gradually their differentiation potential and multipotency, the length of the telomeres is shortened and they undego morphological alterations (Kassem et al., 2004; Bonab et al.,2006). Therefore it is highly recommended to perform genomic monitoring prior the use of MSCs for clinical applications (Wang Y et al., 2013)

1.2.2. Secretion of bioactive factors

Mesenchymal stem cells communicate among them and their environment through the production and secretion of factors and molecules such as soluble proteins, cytokines, free nucleic acids, lipids and extracellular vesicles (Beer et al., 2017).

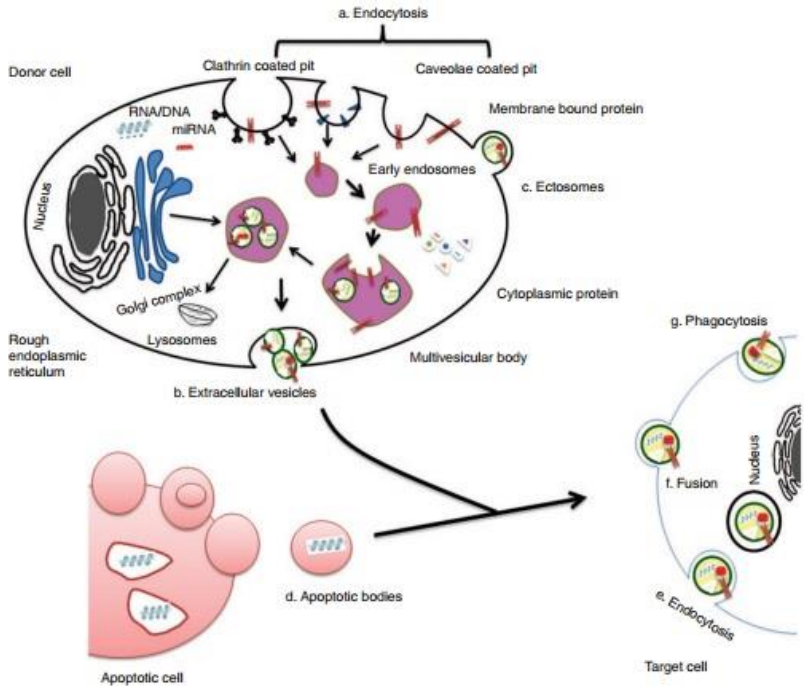

Fig 3: Production of extracellular vesicles *(Rani et al., 2015)*

Extracellular vesicles (EVs) are bilipid membrane vesicles that can vary from 20 – 1000 nm in diameter. They present a mean of inter-cellular communication and contain proteins, lipids and nucleic acids (mRNA and miRNA). EVs are released into the extracellular space from where they enter in the blood vessels or into other biological fluids.

Based on their dimensions and origin, EVs are classified in three categories (Fig.3):

- ectosomes or microvescicles (200-1000nm) that derive from the cellular membrane;
- exosomes (40-100nm) emerging from the inside budding of the late endosomal membrane;
- apoptotic bodies (50-500nm) released from apoptotic cells.

(Hannafon and Ding, 2013; Dragovic et al 2011; Kalra et al., 2012; Mathivanan et al., 2012)

The content of the MSCs secretome varies among different cells and tissues, and are influenced by the physiological and pathological conditions of their environment (Vizoso et al., 2017). The produced molecules are responsible for the beneficial activities of MSCs such as immunomodulation and anti-inflammatory activity; anti-apoptotic activity; wound healing and tissue repair; neuroprotective and neurotrophic effects; angiogenesis regulation; antitumor effect and antimicrobial effect.

- Immunomodulation and anti-inflammatory activity

MSCs are able to modulate the proliferation, activation and function of all immune cells (Fierabracci et al. 2016). The balance between the production of anti-inflammatory cytokines and pro-inflammatory cytokines determines the final immunomodulation effect. Therefore MSCs have been studied for the potential application for the treatment of graft versus host disease (Le Blanc et al. 2004; English et al., 2010), systemic lupus erythematosus (SLE), diabetes mellitus type I, multiple sclerosis and Crohn's disease (Fierabracci et al., 2016).

- Wound healing and tissue repair

The beneficial role of MSCs in wound healing and tissue repair firstly has been attributed to their ability to engraft and differentiate in the required specific cell type. Later several animal models demonstrated the major impact of the autocrine or paracrine effects on the regeneration of the damaged tissues and organs (Salgado et al., 2015, Stastna and Van Eyk 2012; Drago et al., 2013). The stimulation of the proliferation, together with anti-fibrotic and angiogenic factors part of the secretome that reduce the scar formation make MSC suitable for the regenerative medicine (Cargnoni et al. 2012; Preda et al., 2014)

- Angiogenesis regulation

Angiogenesis is an important step of the wound healing process. MSCs have a dual positive effect on the angiogenesis by promoting the proliferation and migration of endothelial cells which results in new vasculature formation and in the prevention of endothelial cell apoptosis demonstrated *in vitro* (Burlacu, 2013). Given the presence of blood vessel abnormalities in a large array of pathologies including insufficient or excessive vessel growth, atherosclerotic diseases and wound healing disorders, MSCs could have a potential clinical therapeutic application. The MSCs secretome contains a balanced mix of angiogenic stimulators and inhibitors secreted into the extracellular space and influenced by the environment i.e. chemokines concentration and hypoxic conditions (De Luca et al., 2011). Several animal models highlighted the successful application of MSCs in cerebral ischemia, myocardial infarction, neurogenic bladder, peripheral artery disease and stress under urinary incontinence (Hsieh et al., 2013; Sharma et al., 2013).

- Neuroprotective and neurotrophic effects

Studies on nerve injury models demonstrated that the production of neuroprotective and neurotrophic factors by MSCs, have a direct effect on the inflammation site by promoting the vascularization and the increment of the myeilin sheaths thickness, modulating the Wallerian degeneration stage, accelerating the fiber regeneration, reducing the fibrotic scaring and improving the fiber organization (Caseiro et al., 2016). Furthermore, the MSCs secretome plays an important role in the neurogenic niche by the secretion of a set of growth factors with favorable effects on neural stem cells (Salgado et al., 2015).

- Antitumoral effect

The role of MSCs on the tumor formation and progression is double, as both pro- and anti-tumor effects have been described. MSCs produce factors and molecules that indirectly influence the tumor or themselves can go under a malignant transformation (Wong, 2011; Houthuijzen et al., 2012). It has been suggested that MSCs isolated from different tissues produce a different set of molecules which influences the final tumoral effect as site-specific functions have been described. Finally, the controversial effect of MSCs can be attributed to the lack of specific markers possibly related to MSCs population with specific effects on tumor progression (Vizoso et al., 2017).

- Anti-apoptotic activity

The production of inhibitor proteins and the influence on the lower expression of anti-apoptotic proteins consents to MSCs to prevent cell death (Li et al., 2015). However it has been reported that MSCs can exert an opposite effect by decreasing pro-apoptotic factor Bax and cleavage of

caspase-3 expression, while increasing the expression of pro-angiogenic factors like basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Tang et al., 2005).

- Antimicrobial effect

MSCs modulate the phagocyte activity by enhancing their action and can be used for the treatment of bacteria – induced sepsis (Gupta et al. 2007; Gonzalez-Rey et al., 2009; Nemeth et al., 2009; Mei et al., 2010; Krasnodembskaya et al.,2010). Precisely, the MSCs secretome contains chemokines such as CXCL10, CXCL8, CXCL1, CXCL6, CCL20, and CCL5, known for the antibacterial effect against *E. coli* and some strains of *Staphyloccocus* (Egesten et al., 2007, Linge et al., 2008). Indeed, in vitro studies have demonstrated the bactericidial effect of MSCs on *E.coli* and *S.epidermidis* (Vizoso et al., 2017).

1.2.3. Immunomodulation

The immunomodulation properties of MSCs make them an important element of cell-based therapies. The underlying 5 main mechanisms are mediated by secretion of factors and by cell to cell contact with immune cells:

(1) regulation of the balance of Th1/Th2 cells and suppression of the proliferation, cytokine secretion and cytotoxicity of T cells (Puissant et al., 2005; Yanez et al., 2006 and Glennie et al., 2005);

(2) regulation of the functions of regulatory T cells (Tregs) (Selmani et al., 2008);

(3) incrementation of the viability of B-cells, however they can also inhibit their proliferation and block the cell cycle. Additionally, MSCs have an effect on the secretion of antibodies and production of co-stimulatory molecules of B cells (Corcione et al., 2006);

(4) inhibition of the maturation, activation and antigen presentation of dendritic cells (Burchell et al., 2010; Ramasamy et al., 2007),

(5) inhibition of the interleukin-2 (IL-2)-induced natural killer (NK) cell activation (Spaggiari et al., 2006).

The immunomodulatory effect of MSCs is influenced by the local microenvironment or by the characteristics of the disease. In case of autoimmune diseases and acute graft *versus* host disease (GvHD), MSCs decrease the Th1 immune response (Le Blanc et al., 2004; Rafei et al., 2009). On contrary, during airway allergic inflammatory diseases such as allergic rhinitis (Cho et al., 2009) and asthma (Goodwin et al., 2011), BM-MSCs shift the immune response from Th2 to Th1.

1.3. Homing and migration of MSCs

Homing is defined as an active or passive arrest of MSC within the vasculature with the subsequent transmigration across the endothelium (Karp and Teo, 2009). The ability to home towards the injury and inflammation site, made MSCs interesting for therapeutic purposes. MSCs can be administered locally or via systemic routes, which leads to the distinction in two types of homing: nonsystemic homing and systemic homing (Fig. 4).

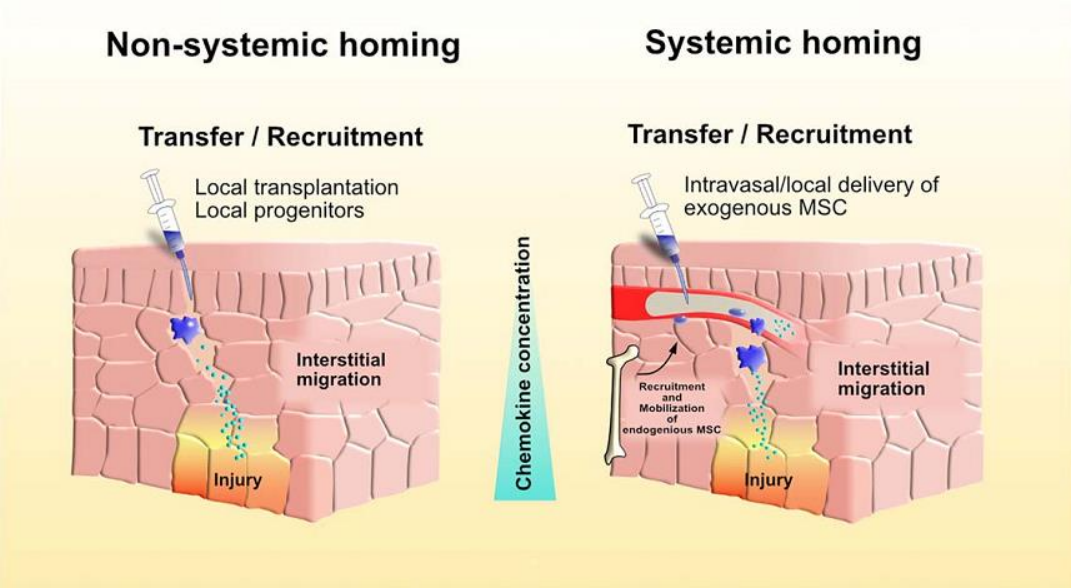

Fig 4: Routes of MSCs homing *(Nietzsche et al, 2017)*

Non-systemic homing requires the presence of the cells in proximity of the lesion area, which means that local MSCs are involved or exogenous MSCs are injected in the target site. MSCs follow a chemiotactic gradient for a direct migration towards the injury during which they are activated and undergo polarization with the formation of a front pole. The role of the last one is to guide the interstitial migration of the cells by sensing the chemokine signals produced at the lesion site.

Systemic homing is a complex process composed of three steps (Fig. 5):

(1) direct administration in blood vessels or cell recruitment with the following ingress into the circulation;

(2) extravasation near the lesion site;

(3) migration in the interstitial tissue to reach the target.

During the 1st step, MSC migrate to the circulation from endogenous niches of the bone marrow or exogenous MSCs are being administered via a systemic route. Through the blood stream, MSCs arrive to the lesion site guided by a gradient of chemokines. As they have reached the target site, MSCs slow down by the interaction of surface molecules with ligands expressed on endothelium cells. However, as an alternative, MSCs can also passively arrest within small-diameter blood vessels and capillaries. The active and passive arrests have different effect on the blood flow. In the first case there are no local blood flow impairments as the cell slowly modifies its shape by flattening. On the contrary, during the passive arrest the shape of cell remains the same, leading to blood flow disturbances (Nitzsche et al., 2017). In fact, one of the main risks of the systemic administration of MSCs is the non-specific passive arrest in small blood vessels in

the lungs, spleen, brain and liver with the risk of microembolism (Chamberlain et al., 2011; Teo et al., 2015; Everaert et al., 2012 and Mitkari et al, 2014). The next phase involves the transendothelial migration (TEM) which allows the MSCs to reach the perivascular space. The lesion site is reached by a final trans-tissue and or interstitial MSCs migration following a chemokine gradient released from the lesion site (Laird et al., 2008).

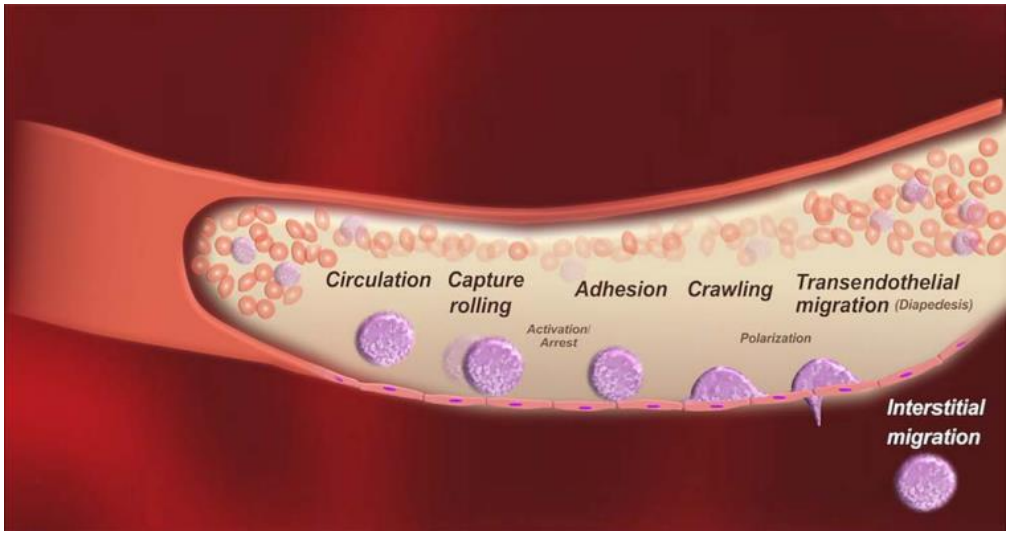

Fig 5: Multi-step cascade of MSCs systemic homing *(Nitzsche et al, 2017)*

1.3.1. Homing of endogenous MSCs

Endogenous MSCs are located within the bone marrow or in the perivascular niche from where they migrate towards the lesion site following their mobilization (Meirelles et al 2006). Two possible hypotheses have been proposed for the recruitment rout of endogenous MSCs. The first one is based on the attraction and migration of the MSCs from the bone marrow through the blood circulation guided by a gradient of cytokines, chemokines, and growth factors released from the injury site. The second one presumes that resident MSCs located in the injured tissue through an interstitial migration or via the microcapillaries reach the target lesioned area (Meirelles et al., 2006).

1.3.2. Homing mechanisms

a. Cell capture and rolling

- Selectin-mediated rolling

Selectins are cell surface receptors expressed on blood vessels that bind to ligands present on the surface of leukocytes causing the tethering, rolling and deceleration of passing leukocytes (Muller, 2011). Although MSCs lack of specific selectin ligands, they express glycoproteins that interact with the P-selectin that can be considered as alternative selectin ligands (Yilmaz et al., 2011). Additionally, platelets may play an important role in the MSCs P-selectin interaction, as it has been demonstrated that platelets or neutrophil-platelet clusters mediate endothelium adhesion (Teo et al., 2015).

- Integrin-mediated rolling

Integrins are transmembrane receptors formed of an α- and β- subunit. MSCs express a range of integrins including β1, β2, α1, α2, α3, α4, α5, α6, and αV (Semon et al., 2010; Ip et al., 2007). The noncovalent bond between the subunits α4 and β1 forms the ligand VLA-4 which plays an important role in the endothelial rolling and arrest at the inflammation sites (Fig. 6). The β1 subunit binds to VCAM-1 and extracellular matrix (ECM) ligands, which is important for the rolling and firm adhesion of MSCs as it has been demonstrated that the specific blocking of β1 significantly reduced the homing abilities (Aldridge et al., 2011). Different isolation and cultivation methods influence the expression of α4, modifying the adhesion ability of MSCs (Ruster et al., 2008; Schmidt et al., 2006a; Semon et al, 2010). However, the induced overexpression of the α4 subunit resulted in bone homing enhancement and engraftment of MSCs transplanted in mice. Since the subunit β1 is sufficiently expressed in MSCs, the complementary bond with the α4 subunit leads increased expression of VLA-4 (Kumar and Ponnazhagan, 2007).

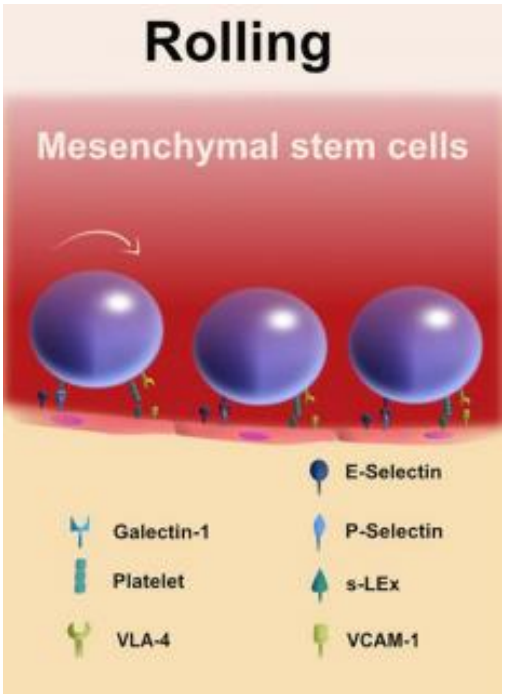

Fig 6: MSCs cell rolling *(Nietzsche et al, 2017)*

b. Cell activation and firm adhesion to the endothelium

The interaction between chemokines and their receptors promotes direct migration, integrin mediated adhesion, and rearrangement of the cytoskeleton of MSCs (Springer, 1994). MSCs express on their surface a broad spectrum of chemokine receptors such as C-C (CCR2, CCR4, CCR7, CR10) and C-X-C chemokine receptors (CXCR5, CXCR6, and CXCR4) (Andreas et al., 2014; Ringe et al., 2007; Wynn et al., 2004). The precise role of chemokine receptors in the extravasation is still not known, however contradictory results have been published. For instance, CXCR4 plays an important role in MSCs homing according to several authors (Marquez-Curtis et al., 2013; Wynn et al., 2004; Kitaori et al., 2009; Vanden Berg-Foels, 2014; Saito et al., 2014). On the other hand, Ip et al. demonstrated that CXCR4 has no direct contribution of TEM in MSCs (Ip et al., 2007). The different statements can be a result of different methods of cell

handling, isolation, and cultivation conditions. The bond between chemokines and their corresponding cell surface receptor activates GTP-ases that promote the allosteric switch and exposure of their extracellular binding site of the VLA-4 receptor. This form of the integrin receptor promotes receptor clustering, lateral movement, migration, adhesion, or diapedesis.

c. Endothelial scanning, crawling, and cell polarization

The movement of MSCs in close contact with the inner blood vessel wall conditioned by chemokine gradients is defined endothelial crawling. The chemokine binding triggers inside-out integrin activation, outside-in integrin signaling and cytoskeletal remodeling. The integration of these elements is the basis of endothelial crawling (Shulman et al., 2009). In particular, the cytoskeletal remodeling consists in the reorganization of the actin filaments leading to cell polarization with the formation of filopodia and pseudopodia (Chamberlain et al., 2011). In proximity with the endothelium MSCs form bleb-like protrusions on their surface which promotes their transendothelial migration (Teo et al, 2012) (Fig.7).

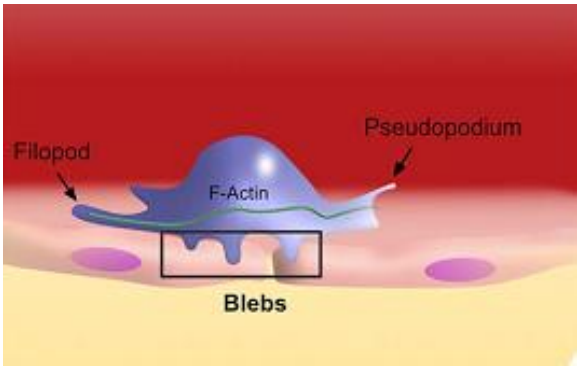

Fig 7: Formation of protrusions promoting MSCs migration *(Nietzsche et al, 2017)*

d. Crossing the endothelial barrier

Each cell that transmigrates has to cross three different barriers: endothelial cells, basement membrane, and pericyte layer (Ley et al., 2007). The interaction between MSCs and the endothelial cells is strictly coordinated by different mechanisms with the final aim to guide the cells through the endothelial junction. Firstly, the formation of transmigratory cups, a form of finger-like endothelial protrusion, surround the adherent MSCs and provide directional guidance (Teo et al.,2012). Furthermore, the bleb-like protrusions of MSCs additionally facilitate the transmigration by mediating intercellular forces against the endothelium (Teo et al., 2012; Ebrahim and Leach, 2015). The activity of Rho mediates the cytoskeleton activation and modulates the MSCs transmigration. Precisely it has been demonstrated that the inhibition of Rho leads to major number of cytoplasmataic protrusions and enhanced chemotactic migration (Jaganathan et al., 2007) (Fig. 8).

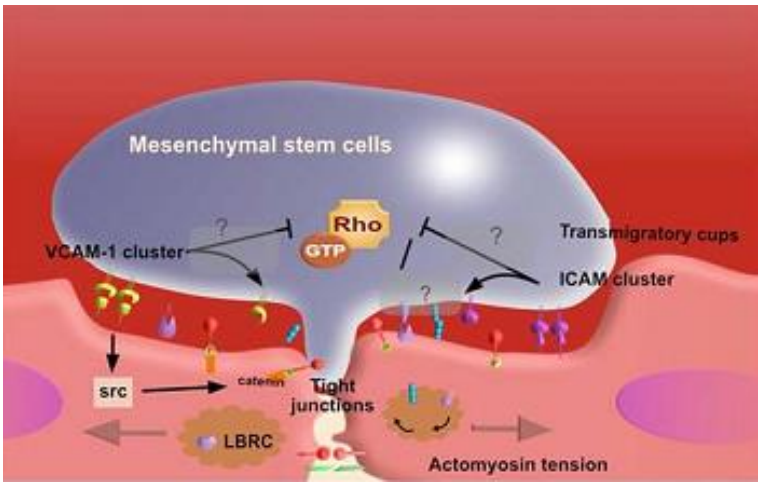

Fig 8: Transmigration of MSCs (a) *(Nietzsche et al, 2017)*

Once the cells are located in the subendothelial layer, they have to cross the basement membrane. The main components of the basal membrane are laminins and collagen type IV. The passage through the basement membrane is facilitated by the production of proteolytic enzymes. Precisely, MSCs produce matrix-metalloproteinases, such as matrix-metalloproteinases 2 (MMP-2), which are able to degrade collagen type IV, and tissue inhibitor of metalloprotease 3 (TIMP-3) (De Becker and Van Hummelen, 2007). Regarding the passage through the pericyte sheath, the exact mechanisms have not been clarified yet. Since leukocytes follow similar transmigration patterns, it is believed that ICAM-1 expressed on pericytes and pericyte-borne chemoattractants possibly guide the final phase of the transmigration (Stark et al., 2013) (Fig. 9).

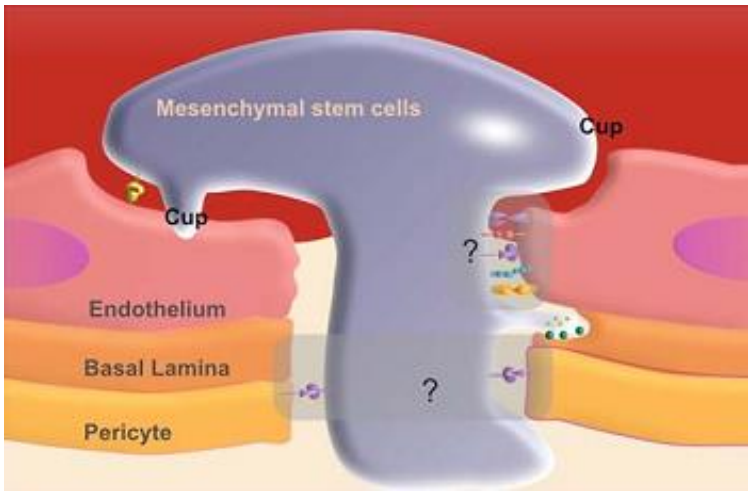

Fig 9: Transmigration of MSCs (b) *(Nietszche et al., 2017)*

1.3.3. Conditioning factors of MSCs homing

- MSCs characteristics and dosage

The *in vitro* expansion of MSCs influences their *in vivo* behavior. Cells with a higher number of *in vitro* passages subsequently have a lower engraftment efficacy. Additionally the homing capacity gradually decreases with the increase of culture time, as freshly isolated MSCs have a greater homing ability (Rombouts and Ploemacher, 2003). The age of the donor influences the characteristics of MSCs. It is known that with aging the ability of the organism to restore its functions and to heal is reduced. This aspect is due to the decreased potency of resident stem/progenitor cells. The additive effect of the in vitro manipulation leads to decreased potency and lower expression of cell surface ligands involved directly in the homing of MSCs (Sahni and Verfaillie, 2013).

- Source and culture conditions of MSCs

MSCs have been isolated from multiple tissues and due to the different native microenvironments in each of them, there are differences in the phenotype of MSCs which influences their use for therapeutic purposes. Culture methods influence the characteristics of MSCs, among them the expression of molecules involved in cell homing. The CXCR4 chemokine receptor is highly expressed on MSCs, however is lost upon culturing. By adding cytokines such as HGF, SCF, IL-3 and IL-6 and under hypoxic conditions the expression of CXCR4 is restored (Wynn et al., 2004; Phinney and Prockop, 2007; Shi et al., 2007; Schioppa et al., 2003) . Additionally it was demonstrated that in different culture systems such as three-dimensional aggregates or spheroids, the functional expression of CXCR4 is restored (Potapova

et al., 2008). The culture conditions such as pO_2, concentration of growth factors, cytokines and media supplements, can regulate the expression of molecules involved in the MSCs homing. For example, higher cell density, hypoxic conditions and inflammatory cytokines such as Il-1β, TNF-α and TGF-1β, up-regulate the expression of matrix metallo-proteasis (MPPs), involved in cell migration (Ries et al., 2007; De Becker et al, 2007).

1.3.4. Delivery of MSCs

In the clinical setting, there are two possible administration routes of MSCs:

- systemic (intravenous [IV] or intra-arterial [IA])
- local administration (intracoronary injection [IC] or local administration in the injured tissue).

In order to obtain a successful therapeutic effect it is necessary to find the optimal route to ease the homing of the MSCs.

Intravenous administration is the most common choice since it offers several advantages: minimal invasiveness with the chance to repeat infusions and maintaining the MSCs close to oxygen- and nutrient- rich vasculature after the homing and transmigration in the lesioned tissue (Sarkar D et al., 2011). The risk of the IV route is the entrapment of the cells in the capillary beds of highly vascularized tissues, such as the lungs (Perreira et al., 1998; Scherepfer et al., 2007).

As an alternative the IA route was studied, since it allows a more localized delivery of MSCs with demonstrated beneficial effects in case brain and subacute spinal cord injury (Walczak et al., 2008; Sykova et al., 2006). However, it is a more invasive procedure and *in vivo* studies

demonstrated higher mortality rate compared to IV route (Lee et al., 2010), possibly due to arterial microvascular occlusions (Walczak et al., 2008). Local administration of MSCs is highly invasive as it requires a surgical intervention, like in case bone fractures or damaged skeletal muscles (Bacou et al., 2004). Additionally the cells are being injected in injured tissues where the local microenvironment is hostile as lack of oxygen and nutrients, hampering the success of the cell therapy (Sarkar D et al., 2011).

1.3.5. Host receptability

During injuries host cells release a set of chemokines that have a positive effect on the homing of MSCs. In fact, in a model myocardial injury, MSCs homed more efficiently 24h post-injury than after two weeks. Therefore strategies have been developed to increase the homing, such as preconditioning in order to express receptors involved in the migration. From the other side, the contrary approach was studied by pre-treating the host with vasodilators to increase the passage of MSCs, avoiding their entrapment in the lungs and allowing a better distribution (Schrepfer et al., 2007).

Chapter 2: Immunophenotypic characterization of adipose tissue derived canine mesenchymal stem cells

2.1. Background: MSCs in veterinary medicine

Companion animals play an important role in the advancement and development of stem cell therapies, since they have been recognized as important translational models of human diseases. The major advantages compared to laboratory animals are: the longer lifespan; the interaction with external and environmental factors involved in the ethiopathogenesis of different pathological states (diabetes, cancer and obesity) and their vulnerability to traumatic injuries similar as humans. The assessment and the follow-up is simpler with the development of imaging techniques for veterinary patients and the biological sampling is more convenient compared to lab animals. Additionally, in the last decades there is a high demand for the development of sophisticated treatments in veterinary patients (Volk and Theoret, 2013). Therefore a more profound knowledge of the stem cells used in veterinary practice and in translational studies is critical for the advancement of the fields of tissue engineering and regenerative medicine.

In companion animals, MSCs are the best characterized adult stem cells because of the wide range of applications form their use for treatments of different clinical conditions in veterinary patients to pre-translational models of human and companion animal diseases.

MSCs have been successfully isolated from dogs and used in the veterinary clinical practice and in pre-translational clinical models of human and companion animal diseases. Similar to human MSCs, the homologue cells isolated from companion animals have a fibroblast-like shape,

adhere on plastic surface, are multipotent and express a set of surface markers. For the reliability of research studies dealing with biological properties of MSC and for the safety of clinical treatments based on these cells, it is necessary to accurately characterize the cell populations. Therefore the aim of this experiment was two-fold:

(1) to design a panel of canine species specific antibodies able to identify cell surface markers defining MSCs (Dominici et al., 2006) that can be readily useful for an appropriate immunophenotypic characterization of canine MSCs;

(2) to evaluate and compare the immunophenotypic profile of canine adipose derived MSCs (AT-MSCs) isolated from subcutaneous and perivisceral adipose tissue, using the above defined panel of antibodies

Taking in consideration the value of dogs as animal models for translational studies and the need to develop cutting-edge veterinary therapies, there is a need to improve the basic knowledge of canine MSCs.

2.2. Materials and methods

2.2.1. Animals

Eight client owned canine patients belonging to different breads, 3 males and 5 females, aged between 1,5 and 12 years, weighing from 4 to 60kg were enrolled in this experiment as adipose tissue donors during scheduled surgical procedures (Table 1). Each patient underwent routine clinical, biochemical and hematological examination prior the surgery. All procedures and protocols were performed by licensed veterinary surgeons, under standard ethical and sterile conditions. The owners of the canine patients signed an informed consent and agreed on the enrollment of their dogs in our study.

Patient	Gender	Age	Breed	Weight	Sampling collection site
Dog 1	Female	1.5 years	Mixed-breed	10kg	Tela subserosa of the perimetrium
Dog 2	Male	9 years	German shepherd	35kg	Abdominal subcutaneous adipose tissue
Dog 3	Female	8 years	Newfoundland	60kg	Mesenteric adipose tissue
Dog 4	Female	12 years	Mixed-breed	30kg	Abdominal subcutaneous adipose tissue
Dog 5	Female	6 years	Mixed-breed	23kg	Tela subserosa of the perimetrium
Dog 6	Male	2 years	Mixed-breed	4kg	Falciform ligament
Dog 7	Female	11 years	Mixed-breed	8kg	Abdominal subcutaneous adipose tissue
Dog 8	Male	4 years	German shepherd	40kg	Abdominal subcutaneous adipose tissue

Table 1: Donors of adipose tissue samples

2.2.2. Sample collection

Adipose tissue samples weighing 1gr were transferred into a tube containing 20ml of Dulbecco's Eagle Modified Medium (DMEM low glucose, Gibco), penicillin (0,10 U/ml), streptomycin (0, 10µg/ml) and amphotericin B (2,5 µg/ml, Gibco). MSCs were isolated after a time range of maximum 4 hours.

Peripheral blood samples for the isolation of peripheral blood mononuclear cells (PBMCs) were collected from 3 healthy dogs' prior orthopedic surgeries. All the samples were collected under the signed informed consent of the owner, under standard ethical and sterile conditions. PBMCs were used as a positive control for the flow cytometrical analysis.

2.2.3. Cell culture

MSCs were isolated from samples of subcutaneous adipose tissue (sAT-MSCs) and perivisceral adipose tissue (pAT-MSCs), weighing 2 grams. Each sample was cut and minced with scalpels in small pieces of 0.5 cm diameter. All of them were transferred in a 15ml conical centrifuge falcon tube containing 10ml of collagenase solution (DMEM low glucose, Gibco; penicillin 0.10 U/ml Gibco; streptomycin 0,10µg/ml, Gibco; amphotericin B 2.5 µg/ml, Gibco and collagenase type I, 0.1% P/V) for an enzymatic digestion in a mechanical stirrer at 37°C for 45 min. The digested sample was centrifuged (210 x *g*/ 8 min) and the collagenase solution and adipose tissue supernatant were removed. The remaining cell pellet was resuspended in 500 µl maintenance medium (DMEM low glucose, Gibco; 10% fetal bovine serum (FBS), Gibco; penicillin 0.10 U/ml Gibco; streptomycin 0.10 µg Gibco, amphotericin B, 2,5 µg/ml Gibco) and seeded in 25 cm2 flasks (Orange Scientific Tissue Culture Flasks) containing 5ml of DMEM in an humidified

incubator at 37°C and 5% CO_2. Medium was changed each 2-3 days. The cells were cultured until they reached 80% confluency and were trypsinized with 0.05% Trypsin-EDTA solution (Gibco). Cells were cultivated until passage 3 (P3) and used for the differentiation studies, flow cytometrical analysis and RT-PCR.

2.2.4. Tri-lineage cell differentiation

a. Adipogenic differentiation

MSCs isolated from subcutaneous and perivisceral adipose tissue, at P3 were seeded in 6 well multiplates at a density of $6x10^3$cells/cm^2. In each well, 3ml of DMEM were added and cells were incubated at 37°C in an incubator with humidified atmosphere of 5%CO_2. As the cells reached 80% confluency, they were treated with adipogenic differentiation media (StemPro Adipogenesis Differentiation Kit) and incubated at 37°C in humidified atmosphere of 5% CO_2. The medium was changed each 2-3 days. After 21 days, the cells were fixed with 70% ethanol and stained with Oil Red O staining.

b. Chondrogenic differentiation

MSCs deriving from subcutaneous and perivisceral adipose tissue, at P3 were seeded in 6 well multiplates at a density of $6x10^3$cells/cm^2. In each well, 3ml od DMEM were added and cells were incubated at 37°C in an incubator with humidified atmosphere of 5%CO_2. As the cells reached 80% confluency, they were treated with chondrogenic differentiation media (StemPro Chondrogenesis Differentiation Kit) and incubated at 37°C in humidified atmosphere of 5% CO_2. The medium was changed each 2-3 days. After 21 days, the cells were fixed with 4% formaldehyde and stained with Alcian Blue staining.

c. Osteogenic differentiation

MSCs deriving from subcutaneous and perivisceral adipose tissue, at P3 were seeded in 6 well multiplates at a density of $6x10^3$cells/$cm^{2.}$ In each well, 3ml of DMEM were added and cells were incubated at 37°C in an incubator with humidified atmosphere of 5%$CO_{2.}$ As the cells reached 80% confluency, they were treated with osteogenic induction media (DMEM with 100nM dexamethasone, 10μM glycerophosphate and 0.250mM ascorbic acid) and ncubated at 37°C in humidified atmosphere of 5% CO_2. The medium was changed each 2-3 days. After 21 days, the cells were fixed with 1% paraformaldehyde and stained with von Kossa staining (Bio Optica).

2.2.5. Immunophenotyping

The assessment of surface marker expression on MSCs isolated from both subcutaneous and perivisceral adipose tissue, was made by single color flow cytometry. Precisely, the expression of the following markers was evaluated: CD29, CD34, CD44, CD45, CD73, CD90 and MHC-II, using a panel of seven antibodies (Table 2).

Prior the analysis of MSCs, the reactivity of each antibody was tested on peripheral blood mononuclear cells (PBMC) isolated from 2ml of peripheral blood of 3 healthy dogs. The blood samples were stratified by density gradient in Histopaque-1077 solution (1:1, v/v, Sigma, St. Louis) and centrifuged at 400xg for 30 min. Purified PBMC were washed with sterile PBS (Sigma) supplemented with 1% fetal FBS. Finally they were resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 2mM L-glutamine, 100mM non-essential amino-acids, 50mM 2-mercaptoethanol (Sigma) and 100 U/ml penicillin G, 100μg streptomycin and 0,25μg/ml amphotericin B. The cells were counted and their concentration was assessed prior the FACS analysis. 4x105 cells were used for the testing of each antibody (5μl/sample). After a 15 min dark incubation at RT, the cells were washed with 1% FBS, centrifuged for 5 min at 400xg, resuspended in 0.5ml of PBS/1%FBS and finally set aside for FACS analysis (Cytomics FC 500, Beckman Coulter).

Cell marker	Clone	Isotype	Antibody label	Reactivity	Production company
CD 29	TS2/16	IgG1,k	PE	Human;dog; bovine	BioLegend
CD 34	1H6	IgG1	PE	Dog	eBioscience
CD 44	YKIX337.8	IgG2a,k	FITC	Dog	eBioscience
CD 45	YKIX716.13	IgG2b,k	FITC	Dog	eBioscience
CD 73	Polyclonal	IgG	Alexa Fluor 647	Human; mouse; rat; dog; chicken	Bioss
CD 90	YKIX337.217	IgG2b,k	PE	Dog	eBioscience
MHC-II	YKIX337.8	IgG2a,k	APC	Dog	eBioscience

Table 2: Antibodies used for immunophenotypic characterization

MSCs at P3 were trypsinized (0,05% Trypsin-EDTA, Gibco) and centrifuged at 210xg for 8 min. The cell pellet was resuspended in 3ml of complete medium for cell count using a Burker's hemocytometer. For FACS analysis, 2.5 x 10^4 cells were transferred in conical and round bottom tubes, washed with 1ml sterile PBS supplemented with 1% fetal bovine serum (FBS), and finally centrifuged at 210 x g for 8 min. After eliminating the supernatant, in every tube 5 µl of antibody was added. For every sample coming from the same animal, one tube containing the same number of unmarked cells was evaluated as a negative control. Following dark incubation at room temperature (RT) for 15 min, 1ml of PBS was added and cells were centrifuged at 210 x g for 8 min. To each tube 0.5 ml of sterile PBS was added and the samples were evaluated by flow cytometry analysis (Cytomics FC 500, Beckman Coulter). The dead cells were excluded from the analysis using Sytox AAdvanced Dead Cell Stain Kit (Life Tecnologies), according to the manufacturer's instructions.

2.2.6. RT-PCR

Reverse-Transcriptase-PCR (RT-PCR) was performed to evaluate the phenotypic expression of CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and Oct-4 (Table 3). Total RNA was extracted from 1.5 x 10^6 MSCs at P3, using the kit Nucleospin®RNAII (Macherey-Nagel) following the indicated manufacturer's instructions. The retrotrascription of 1.5µg total RNA was made using RevertAit™ First Strand cDNA Synthesis Kit (Fermentas) and the resulting cDNA was used as a template for PCR. The final PCR mix contained 2µl cDNA, 1x amplificiation buffer with 2.5 mM $MgCl_2$, 10 mM dNTP mix (Thermo Scientific), 0.25µm forward and reverse primers and 1 U DreamTaq (Thermo Scientific) in a final volume of 25µl. All PCRs were performed under the following protocol: denaturation step at 94°C for 30 and 35 cycles of annealing at 55°C and extension at 72°C for 30s. The products of the RT-PCR were separated on agarose gel (1,5% P/V) in TAE buffer stained with 3.5µl ethidium bromide (10mg/ml). All amplicons were visualized under UV light with a trans-illuminator and images were acquired by a Canon digital camera. The analysis was repeated with two different replicates for each tissue sample. Semi-quantitative analysis of the expression of the MSCs markers was performed by evaluating the optical density of each positive band by means of ImageJ processing software, normalized to the expression of the reference gene GADPH.

Gene	Accesion number	Primers	Amplicon size
CD13	NM_001146034.1	Fw: GGTCCTTACCATCACCTGGC Rv: CCTAAGGCCATCCATCGTCC	335 bp
CD29	XM_005616949.1	Fw: AGGATGTTGACGACTGCTGG Rv: ACCTTTGCATTCAGTGTTGTGC	356 bp
CD31	XM_848326	Fw: GCCCGAAGTTCACTCTCAAG Rv:CACTCCTTTGACCCACACCT	410 bp
CD34	NM_001003341.1	Fw: GAGATCACCCTAACGCCTGG Rv: GGCTCCTTCTCACACAGGAC	383 bp
CD44	NM_001197022.1	Fw: CCCATTACCAAAGACCACGA Rv: TTCTCGAGGTTCCGTGTCTC	408 bp
CD45	XM_005622282.1	Fw: TGTTTCCAGTTCTGTTTCCCCA Rv: TCAGGTACAAAGCCTTCCCA	432 bp
CD73	XM_532221.4	Fw: GATGGGAAAGGCAAGAGGCT Rv: TTCCTGGCATCTTGCTACGG	317 bp
CD90	NM_001287129.1	Fw: AAGCCAGGATTGGGGATGTG Rv: TGTGGCAGAGAAAGCTCCTG	285 bp
CD105	XM_005625330.2	Fw: GCTGAGGACAGAGATGACCA Rv: CACGGAGGAGGAAGCTGAAG	421 bp
Oct-4	XM_538830.1	Fw: AAGCCTGCAGAAAGACCTG Rv: GTTCGCTTTCTCTTTCGGGC	286 bp
GAPDH	NM_001003142.1	Fw: TTCACCACCATGGAGAAGGC Rv: ACTGATACATTGGGGGTGGG	442 bp

Table 3: Primers used for RT-PCR analysis of MSCs markers

2.2.7. Statistical analysis

The results of the immunophenotypic characterization of MSCs deriving from perivisceral and subcutaneous adipose tissue were compared. Data was expressed as mean±standard deviation (SD). The differences among the groups were considered statistically significant for $P<0.05$ and Mood's median test was made as non-parametric statistics analysis (SPSS 16IBM Software).

2.3. Results

2.3.1. Cell culture and isolation

MSCs from both subcutaneous and perivisceral adipose tissue, were adherent on the surface of the flasks after 24-48 post seeding. During their culture, they appeared morphologically as a homogeneous population of fibroblast-like cells with elongated spindle like shape (Fig. 10)

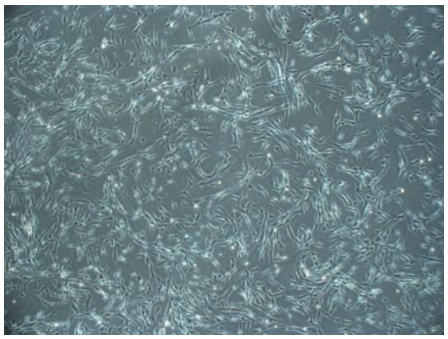

Fig 10: MSCs culture at P3

2.3.2. Tri-lineage cell differentiation

MSCs at P3 from perivisceral and subcutaneous adipose tissue were able to differentiate into osteogenic, adipogenic and chondrogenic lineage. Cells treated with adipogenic medium contained intracellular lipid rich vacuoles stained with Oil Red O, confirming the adipogenic differentiation in vitro. The presence of aggregates and extracellular calcium deposits in cell cultures stimulated with ostegenic induction medium and stained with von Kossa staining, confirmed the osteogenic differentiation in cells from both sources. Finally, MSCs treated with chondrogenic medium contained aggregates of proteoglycans stained in blue with Alcian Blue staining. The negative control for each differentiation treatment showed no evidence of any differentiation (Fig. 11).

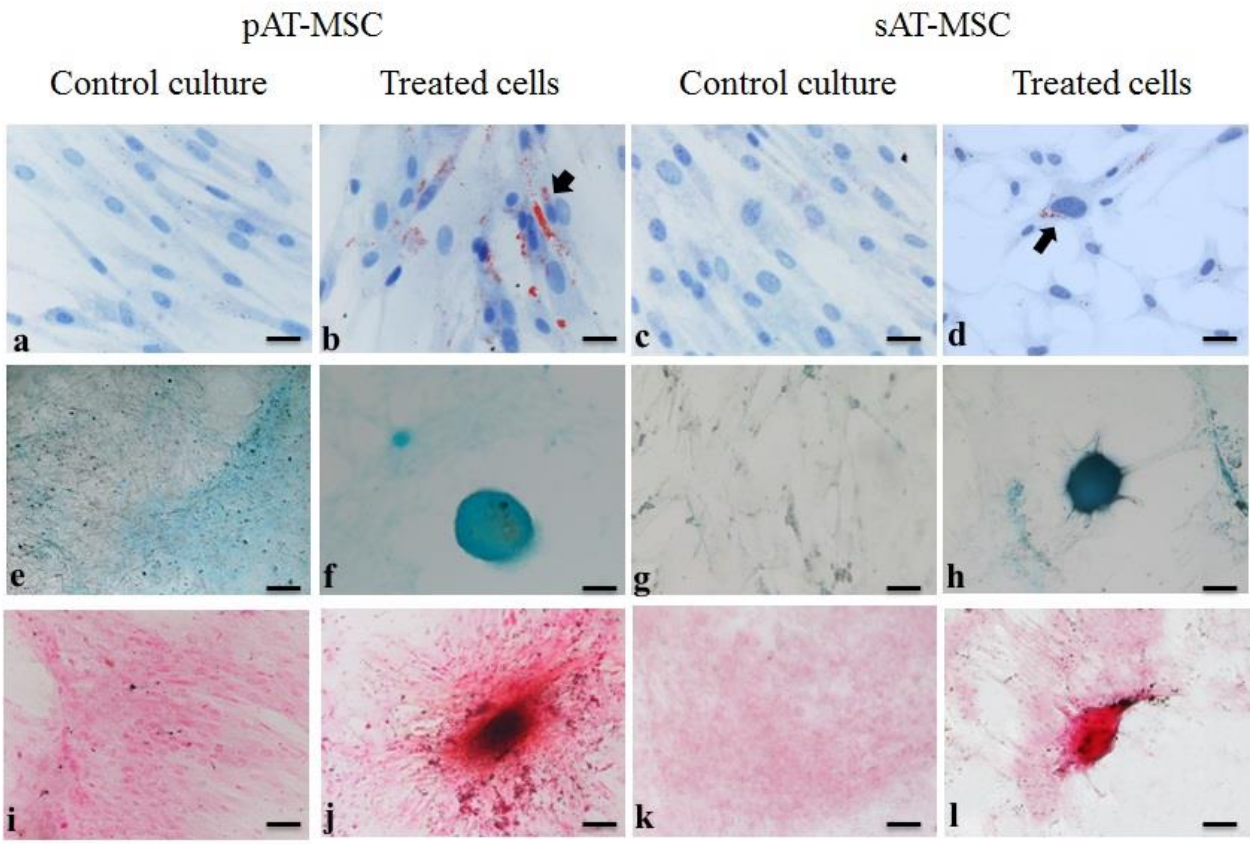

Fig 11: In vitro tri-lineage cell differentiation

Photomicrographs of canine perivisceral adipose tissue derived MSC- (pAT-MSC: b, f, j) and canine subcutaneous adipose tissue derived MSC (sAT-MSC: d, g, l), after 21 days of culture in adipogenic (b, d), chondrogenic (f, h), osteogenic (j, l) induction medium. Parallely a control culture of pAT-MSC (a, e, i) and sAT-MSC (c, g, k)was grown for 21 days in DMEM. Adipogenic differentiation is evidenced with the presence of intracellular vacuoles colored in red (black arrow) with Oil Red O staining, in pAT-MSC (b) and sAT-MSC (d), which are absent in control groups (a, c) (40×, scale bar 200 μm). Alcian blue staining indicated the presence of aggregates of proteglycans present in treated pAT-MSC (f) and sAT-MSC (h), and their absence in the control cultures (e, h) (10×, scale bar 100 μm). Osteogenic differentiation was indicated with extracellular calcium aggregates stained with von Kossa coloration in pAT-MSC (j) and sAT-MSC (l) stimulated with ostegenic medium. Control groups did not contain any calcium aggregates (i, k) (10×, scale bar 100 μm).

2.3.3. FACS analysis

All the antibodies used in the experimented and tested on canine PBMC showed the expected reactivity. The live population of MSCs was gated in the scatter plot for further fluorescence intensity analysis in the histogram plot (Fig.12). The results from the FACS analysis showed that MSCs at P3 from perivisceral and subcutaneous adipose tissue were positive for CD29, CD44, CD73, CD90, and negative for CD34, CD45 and MHC-II (Table 3). There was no significant difference in the surface expression of the markers between the two groups of MSCs.

Markers	**sAT-MSCs**	**pAT-MSCs**
CD29	99.5%±0.6	99.2%±1.5
CD34	0.5%±1	0.25%±0.5
CD44	78%±17	76.7%±18
CD45	0.25%±0.5	0.5%±0.6
CD73	14%±12.3	17%±14
CD90	89%±6.7	79.5±7.1
MHC-II	4.5%±6.3	4.5%±6.4

Table 3: FACS analysis of adipose tissue derived MSCs

The data in displayed in the table are percentages expressed as mean ±SD

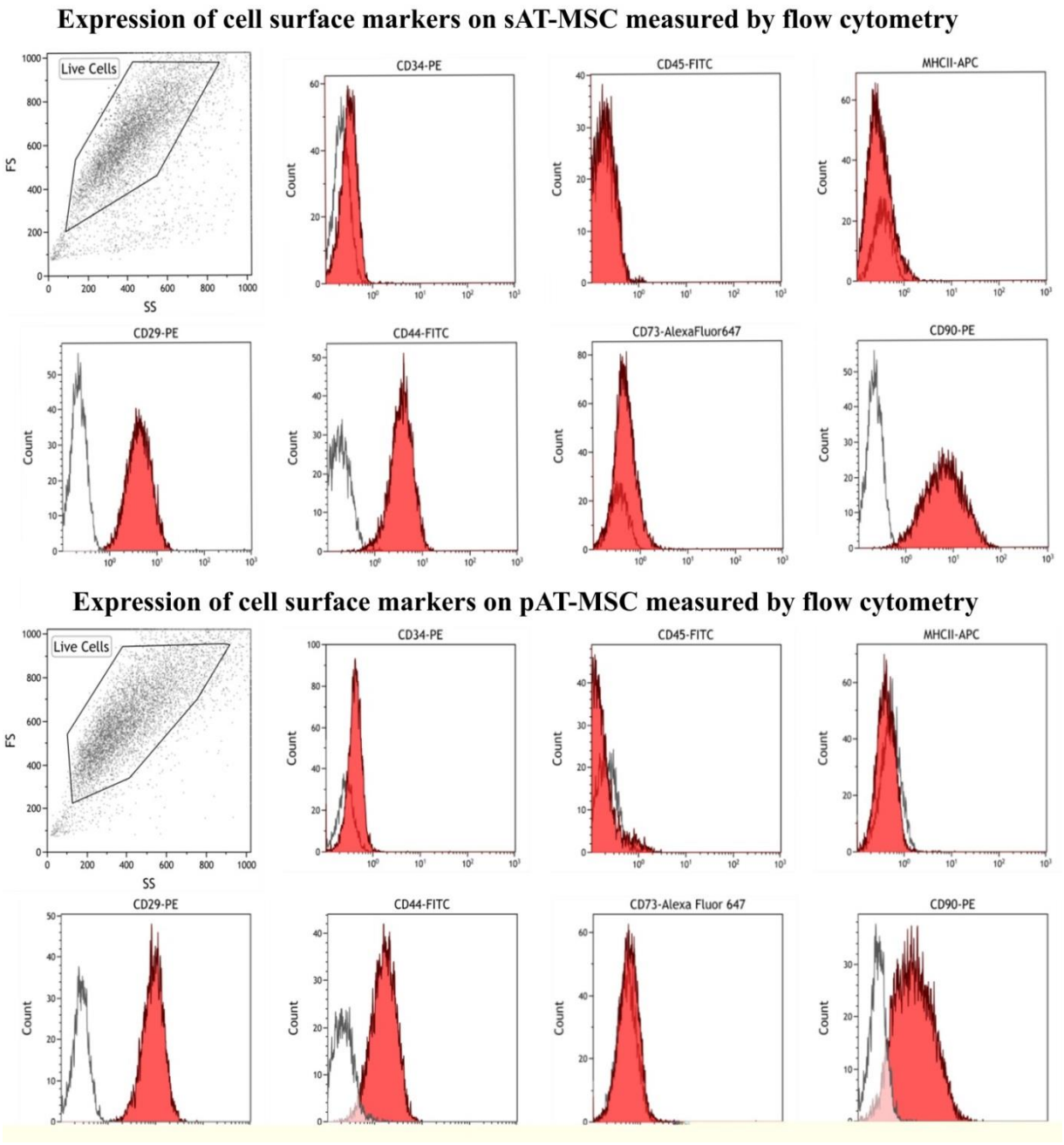

Fig 12: Flow cytometry analysis of the expression of cell surface markers on sAT-MSC and pAT-MSC

The expression of cell surface markers is presented in two panels. The upper panel contains data regarding the MSCs deriving from subcutaneous adipose tissue and the low panel data about MSCs deriving from perivisceral adipose tissue. Live cell populations were gated in forward and side scatter for further analysis. Each histogram contains two peaks. The shaded red peak represents the actual expression of the markers for each cell group, on the other hand the transparent one is the negative control.

2.3.4. Reverse transcription analysis of the gene expression of MSCs markers

The expression of a set of cell marker genes typical for MSCs was performed on sAT-MSCs and pAT-MSCs at P3. The two cell populations resulted negative for CD45 and CD31. Regarding CD34 expression, pAT-MSCs were negative, while sAT-MSCs had a weak expression. The other genes from the panel were positively expressed in both cell populations. Finally, the semiquantitative analysis of the marker expression, demonstrated a similar expression of the genes between the two populations (Table 4).

Gene	**pAT-MSCs**	**sAT-MSCs**
CD90	+++	+++
CD73	++	++
CD105	++	++
CD45	-	-
CD34	-	+/-
CD44	+++	+++
CD13	+++	+++
CD29	+++	+++
CD31	-	-
Oct-4	+	+

- (Not expressed)
+ (<25% of GAPDH signal)
++ (25-50% of GAPDH signal)
+++ (>50% of GAPDH signal)

Table 4: Gene expression of MSCs markers

2.4. Discussion

MSCs based treatment has been increasingly applied in veterinary medicine in the recent years. Several studies have reported the beneficial effects for different diseases in dogs such as chronic osteoarthritis of the elbow joint (Black et al., 2008), canine atopic dermatitis (Hall et al., 2010), dilated cardiomyopathy (Pogue et al., 2013), oral ulcers (Alamoudi et al., 2014), hyp dysplasia (Marx et al., 2014), spontaneously injured spinal cord (Penha et al., 2014), keratoconjuctivitis sicca (Villatoro et al., 2014), inflammatory bowel disease (Perez-Marino et al., 2015), acute toracolombar disease (Kim et al., 2015) and chronic spinal cord injury (Lee et al., 2015). Although the majority of the studies carried out contain a limited number of patients and often lack of appropriate controls, the results can be considered encouraging for the set-up of clinical protocols to be applied in veterinary medicine. For the reliability of research studies dealing with biological properties of MSCs and for the safety of clinical treatments based on these cells, it is necessary to accurately characterize the cell populations. The availability of several possible tissue sources, different methods of cell culture, expansion and handling, as well as a variety of possible therapeutic approaches, represent a limit to the correct evaluation of the healing potential of these cells.

The first aim of this study was to select a panel of species-specific antibodies for the evaluation of cell surface CD marker expression in canine adipose tissue derived MSCs that could be routinely used for the cell characterization prior to their use. Having read previous studies addressing the same topic, we encountered a common statement declared by researchers, saying that their findings can be found unexpected or contradictory due to the low binding affinity of non-species-specific antibodies used for the screening of cell surface markers (Screven et al.,

2014). We hypothesized that by using a panel of species-specific antibodies this variable would be eliminated. The panel would give more reliable and reproducible results that are necessary for the correct characterization of MSCs, since they have become an attractive cell therapy product for small as well as large animal veterinary practitioners.

The second aim was to compare the marker expression of MSCs derived from subcutaneous and perivisceral adipose tissue. Indeed, the characterization of these two populations of MSCs would be of practical relevance for their clinical use. Subcutaneous adipose tissue is an easily available source of MSCs that can be collected quickly and safely during surgery in dogs of different sizes, avoiding the potential risk associated with abdominal surgery or bone marrow aspiration. We hypothesized that by defining and comparing the cell surface pattern of subcutaneous and perivisceral adipose tissue, we could obtain data that will offer researchers and veterinary practitioners the opportunity to choose the source of MSCs for their applications.

MSCs derived from the two tissue sources were analyzed at P3, as this passage is considered appropriate for obtaining an adequate number of cells, safe in terms of chromosome variability and genetic abnormalities, and, therefore, adequate for therapeutic applications (Binato et al., 2013). MSCs from both tissue sources had a similar fibroblast-like morphology, were able to adhere to plastic surface, grew in monolayer and demonstrated the capacity to differentiate in osteogenic, chondrogenic and adipogenic lineages when stimulated with appropriate induction medium. Previous study reported similar results (Guercio et al., 2013), but lacked the immunophenotypic characterization of the cell population. Thuerefore, I chose to determine the expression of a panel of cell surface CD markers (CD29, CD34, CD44, CD45, CD90, CD73 and MHC-II), typical for MSCs (Dominici et al., 2006) using species-specific anti-canine antibodies.

CD 105 - one of the three surface markers that define human MSCs (Dominici et al., 2006) was not taken in consideration in our study since there are not any commercially available anti canine - CD105 antigen. However by means of RT-PCR the CD105 expression was analyzed and it was found positive in both sAT-MSCs and in pAT-MSCs

Antibody reactivity was confirmed by flow cytometry of canine PBMC that served as a positive control. Interestingly, canine AT-MSCs derived from subcutaneous and perivisceral adipose tissue showed a similar immunophenotypic pattern, which from a practical point of view indicates that both tissues can be used as a valid source for the isolation of MSCs. Both cell populations can be defined as CD29+CD44+CD90+CD45−CD34−CD73− MHC-II−. As far as I know, this is the first study that characterized canine AT-MSCs by using a panel of canine species specific antibodies for both positive as well as negative markers. Different researchers included anti-human antibodies in their protocols, however, they cannot be considered fully reliable for the characterization of canine MSCs. Rozemuller et al. (2010) studied the expression of surface cell surface marker expression on canine BM-MSCs using a panel of 43 anti-human antibodies. 24 of them, among which CD73, CD90 and CD105 did react with human MSCs, but failed to cross-react with canine MSCs, therefore cannot be considered adequate for the definition of the immune phenotype of canine MSCs. Takemitsu et al. (2012), isolated and characterized canine AT-MSCs obtaining surprisingly low results for the expression of CD90 and CD105, finding that can be justified by the fact that they used non canine specific antibodies. Similar findings were obtained by Screven et al. for CD105, as they stated that the low binding activity can be a consequence to the use of a nonspecific canine antibody.

By RT-PCR analysis, the gene expression of the same markers analyzed with FACS analysis, including additionally Oct-4, CD105, CD13 and CD31. The positive expression of Oct-4 demonstrated the pluripotency of both sAT-MSCs as well as pAT-MSCs, as it has already been described in canine (Neupane et al., 2008; Reich et al., 2012) and human (Han et al., 2014) AT-MSCs. Furthermore, the cells resulted positive for CD13 and negative for CD 31. Although we did not evaluate CD105 expression by flow cytometryanalysis,the RT-PCR reveale da positive expression for both sAT-MSCs and pAT-MSCs.

Finally, the results obtained by flow cytometry were confirmed by RT-PCR, as the two cell populations demonstrated a similar qualitative pattern of expression, with the only exception of CD34. A complete overlap of marker expression between human MSCs has yet to be demonstrated due to the lack of an extensive cell characterization in some species. However, similarities can be found between MSCs derived from domestic and laboratory animals and humans. Most of the markers analyzed in this study share a similar distribution in the different species, with the exception of CD73.

We encountered a similar expression of CD29, CD34, CD44, CD45, CD90 and MHC-II which could suggest that in vitro cultured canine MSCs possibly share a similar immunophenotypic profile with human MSCs (Takemitsu et al., 2012; Nery et al., 2013). The unique difference found was in the expression of CD73, one of the three positive markers proposed for defining human MSCs (≥95%) (Dominici et al., 2006).

This would suggest that there is a slightly different immunophenotypic profile that should be taken in consideration for the correct interpretation and characterization of AT-MSCs in veterinary patients (Ivanovska et al., 2017).

Chapter 3: Expression of CXCR4 in canine adipose tissue derived mesenchymal stem cells

3.1. Background: CXCR4/SDF1 axis in MSCs migration

Chemokine receptor 4 (CXCR4) and its ligand, the stromal cell-derived factor-1 (SDF-1) are key molecules involved in the migration of MSCs towards the site of the lesion. The enhanced secretion of SDF-1 at the site of the injury creates a microenvironment that attracts the migration of CXCR4-positive cells (Petit et al., 2007). The importance of the axis CXCR4/SDF1 in the homing of transplanted MSCs has been demonstrated in animal models of acute kidney injury in mice (Tögel F, et al., 2005); post-myocardial infarction treatment (Dong et al., 2012); healing of burn wounds and contribution to the epithelization of wound repair (Hu et al., 2013), and migration to neo-angiogenesis niches with the following re-vascularization of ischemic tissues in case of acute ischemia (Petit et al., 2007).

For a successful clinical application, a high number of transplanted MSCs is needed; for example in human medicine the dosage is 1-5 x 10^6 cells/kg (Lin and Hogan, 2011). Therefore, MSCs need to be amplified extensively *in vitro* to obtain significant cell numbers readily usable. However, it has been demonstrated that the culturing and passaging of cells modifies their phenotype. The expression of adhesion molecules is reduced, chemokine receptors for CXCL12 (SDF-1), CX3CL1, CXCL16, CCL3, CCL19 and CCL21 are lost which leads to alterations in the homing abilities of MSCs (Honczarenko et al., 2006). As a matter of fact, human and murine MSCs of earlies passages express CXCR4 and migrate in respond to the stimulus SDF-1. Subculturing for more than two passages reduced the expression of CXCR4 and the cells lack chemiotactic response to chemokines. Finally, the impairment of the homing challenges the success of the therapeutic application of MSCs (Wynn et al., 2004, Rombouts et al., 2003).

The aim of this experiment was to study the expression of the receptor CXCR4 in canine adipose tissue derived MSCs. Precisely, to compare the expression of the receptor during different passages in vitro, via FACS analysis and qPCR. Finally, the contribution of the CXCR4/SDF1 axis in the migration of canine AT-MSCs was evaluated in vitro with Transwell chambers. To date, in the literature there are no data about this important aspect of canine AT-MSCs.

3.2. Materials and methods

3.2.1. Animals

Three healthy female Beagle dogs (3-5 years old, 9-12 kg), from the animal house of the National Research Directorate for Radiobiology and Radiohygiene, Budapest, Hungary were involved in this experimental study as adipose tissue donors. The animals had water at disposition ad libitum, were fed regularly and kept in standard light/dark cycle conditions (22-24 ºC; humidity: 55 ± 6%) in compliance with the animal protection and keeping regulations (Ethical license number: 22.1/4200/003/2009).

3.2.2. Adipose tissue sampling

Subcutaneous adipose tissue samples were harvested from subcutaneous adipose tissue at the level of the scapular region, during sterile standard surgical procedure and transferred in a sterile tube containing 15ml of DMEM (Gibco), until cell isolation (<2 h).

3.2.3. MSCs isolation and culture

MSCs were isolated from samples of subcutaneous adipose tissue weighing 1 g . Each sample was cut and minced with scalpels in small pieces of 0.5 cm diameter and washed with sterile PBS. All of them were transferred in a 15ml conical centrifuge falcon tube containing 10ml of collagenase solution (DMEM, Lonza; Pen/Strep stock, 10K/10K Lonza; collagenase type I, 0.1% P/V, Sigma - Aldrich) for an enzymatic digestion at 37°C for 1h. The digested sample was centrifuged (210 x *g*/ 8 min) and the collagenase solution and adipose tissue supernatant were removed. The remaining cell pellet was resuspended in 500 µl maintenance medium (DMEM, Lonza; 10% fetal bovine serum (FBS), Lonza; Pen/Strep stock, 10K/10K Lonza) and seeded in

75 cm2 flasks (Orange Scientific Tissue Culture Flasks) containing 15ml of DMEM in an humidified incubator at 37°C and 5% CO_2. Medium was changed each 2-3 days. The cells were cultured until they reached 80% confluency and were trypsinized with TrypLE™ Select Enzyme (1X) (ThermoFisher Scientific). Cells were cultivated until P3 and used for the flow cytometrical analysis, qPCR and Transwell experiments. Before each experiment, the viability of the cells was evaluated with Trypan Blue Solution (Sigma-Aldrich).

3.2.4. FACS analysis

a) Immunophenotypic characterization

MSCs at P3 were characterized by single color flow cytometry for their expression of surface markers: CD29, CD34, CD44, CD45, CD90 and MHC-II using a set of monoclonal species-specific antibodies (Table 2). Before the analysis, the cells were trypsinized (TrypLe Select, Thermo Fisher Scientific) and centrifuged at 210xg for 8 min. The cell pellet was resuspended in 3ml of complete medium for cell count using a Burker's hemocytometer. For FACS analysis, 2.5 x 10^4 cells were transferred in conical and round bottom tubes, washed with 1ml sterile PBS supplemented with 1% fetal bovine serum (FBS), and finally centrifuged at 210 x g for 8 min. After eliminating the supernatant, in every tube 5 μl of antibody was added. For every sample coming from the same animal, one tube containing the same number of unmarked cells was evaluated as a negative control. Following dark incubation at room temperature (RT) for 15 min, 1ml of PBS was added and cells were centrifuged at 210 x g for 8 min. To each tube 0.5 ml of sterile PBS was added and the samples were evaluated by flow cytometry analysis.

b) CXCR4 expression

Single color flow cytometrical analysis was performed to assess the expression of CXCR4 on MSCs at P0 and P3. Cells were seeded at a density of 5000 cells/cm^2, when they reached the confluency of 80%, were trypsinized with TrypLE™ Select Enzyme (1X) (ThermoFisher Scientific) and counted with Burker's haemocytometer.

Prior the analysis of MSCs, the reactivity the antibody was tested on peripheral blood mononuclear cells (PBMC) isolated from 2ml of peripheral blood of healthy Beagle dogs. The blood samples were stratified by density gradient in Histopaque-1077 solution (1:1, v/v, Sigma, St. Louis) and centrifuged at 400xg for 30 min. Purified PBMC were washed with sterile PBS (Sigma) supplemented with 1% fetal FBS. Finally they were resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 2mM L-glutamine, 100mM non-essential amino-acids, 50mM 2-mercaptoethanol (Sigma) and 100 U/ml penicillin G, 100μg streptomycin and 0,25μg/ml amphotericin B. The cells were counted and their concentration was assessed prior the FACS analysis. $4x10^5$ cells were used for the testing the anti-CXCR4 antibody (2μl/sample). After a 30 min dark incubation at 4°C, the cells were washed with 1% FBS, centrifuged for 5 min at 400xg, resuspended in 0.5ml of PBS/1%FBS and finally set aside for FACS analysis (Cytomics FC 500, Beckman Coulter).

For the analysis of MSCs, in single FACS tubes $1x10^6$ cells of each sample and 1 ml staining buffer containing Hank's Balanced Salt Solution (ThermoFisher Scientific) + 10%BSA (Sigma-Aldrich) were added and centrifuged at 2000rpm/10 min to eliminate the culture medium. After, the cell pellet was suspended in 100 μl of staining buffer and 2μl of primary fluorescence-

conjugated antibody was added (CD184 (CXCR4) Monoclonal Antibody (12G5), PE, eBioscience™), according the manufacturer's instructions. Cells were incubated at 4°C for 30 min in the dark and after were washed with 2 ml staining buffer to remove the residual primary antibody. The control samples ($1x10^6$ cells) included in the study were: (a) corresponding conjugated isotype control antibody (Mouse IgG2a kappa Isotype Control, PE, eBioscience™) and (b) negative control without any antibody. The samples were analyzed using FACSCalibur flow cytometer (Beckton Dickinson, NJ, USA). Analysis was performed using the Cell-Quest Pro data acquisition and analysis software (Beckton Dickinson).

3.2.5. Total RNA extraction and reverse transcription (RT)

MSCs at P0 and P3 seeded in 75 cm^2 flasks (5000 cells/cm^2) in complete growth media (DMEM+10%FBS) and incubated at 37°C in humidified atmosphere (5% CO2) were used for qPCR. Cells were trypsinized with TrypLe Select (ThermoFisher Scientific) and RNA was extracted from samples of resuspended cells from each replicate culture using Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA) following the manufacturer's instructions. The purity and concentration of the total RNA was measured by UV-spectrophotometry at 260/280 and 260nm respectively. The RNA samples were stored at -80°C until retrotrascription.

Total RNA (1.4 μg/20μl) was used for the synthesis of first strand cDNA using the Thermo Scientific Revert First Strand cDNA Synthesis Kit (Thermo Scientific). The reaction was performed by a Applied Biosystem Step One Plus Real Time PCR, according to the manufacturer's indications. The cDNA samples were stored at -20°C until qPCR analysis.

3.2.6. qPCR

The resultant cDNAs were used as templates for real-time quantitative PCR (qPCR). qPCR amplifications of C-X-C chemokine receptor type 4 (CXCR4) and stromal derived factor 1 (SDF-1) were performed. The reference gene ribosomal protein S19 (RPS19), was selected because of the stable gene expression, among other genes highly recommended for canine cells such as β-2-microglobulin (B2M), ribosomal protein S5 (RPS5), and hypoxanthine phosphoribosyltransferase (HPRT) (Brinkhof et al., 2006). The oligonucleotide primers used are based on published canine cDNA sequences (Iohara et al., 2011; Lee et al., 2016) or were designed (NCBI, Primer – Blast) and purchased from Eurofins MWG Operon (Ebersberg, Germany). All primer pairs are positioned on different exons to reduce the chance of amplifying traces of genomic DNA (Table 5). qPCR reactions were performed using 20ng cDNA and 0.3 µM of the primers in a final volume of 20 µl of 2 x Maxima SXBR green/ROX qPCR Mastermix (Thermo Scientific), according to the manufacturer's instructions in an Applied Biosystem Step One Plus Real Time PCR. The cycle parameters were: 50 °C for 2 min, one denaturation step at 95 °C for 10 min and 40 repeated cycles of denaturation at 95 °C for 15 s followed by an annealing step at 60 °C for 30s and elongation at 72 °C for 30 s. At the end of the amplification cycle, a melting curve analysis for specific amplification control was performed. The gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen. Precisely, the relative gene expression of each transcript, normalized to the RPS19 cDNA amount and expressed as relative quantities, was calculated with regards to the expression level in canine AT-MSCs at P0 cultivated in under standard culture conditions. Each experiment was performed in triplicates from three different canine AT-MSCs donors.

Gene	Accession number	Primers	Amplicon size
RPS19	XM_005616513.3	Fw: CCTTCCTCAAAAA/GTCTGGG Rv: GTTCTCATCGTAGGGAGCAAG	95bp
CXCR4	NM_001048026.1	Fw: GGGCGAGCGGTTACCAT Rv: TGCCCA CTATGCCAGTCAAG	184bp
SDF-1	DQ182700	Fw: GCCATGAACGCCAAGGTC Rv: CTTGTTTTAGAGCTTTCTCCAGGT	270bp

Table 5: Primers used for qPCR analysis of CXCR4 and SDF-1

3.2.7. Chemotaxis assay

The *in vitro* migration of MSCs was assessed in Transwell inserts with 8.0 μm diameter pore size (Falcon® Cell Culture Insert, Corning, NY, USA). MSCs at P3 were grown to confluency (80%), then trypsinized with TrypLe Select (Thermo Fisher Scientic) and cultured overnight with serum-free DMEM prior the experiment. 1 x 10^5 cells suspended in 500μl of serum-free DMEM were loaded onto the upper chamber and different chemotactic factors suspended in 500 μl DMEM were load in the bottom chamber. Five different experiments were performed (Fig. 13). After an overnight incubation of 16 hours, all of the remaining (non-migrated) cells were removed with a cotton swab from the upper side of the membrane. The membrane with the migrated cells on the lower part was fixed with methanol and stained with Diff Quick staining. The inserts were washed in distilled water and let dry at RT. After the membrane was gently removed and mounted on microscope slides for cell count. The number of stained MSCs for each condition was counted manually in 5 fields under a light microscope at 10X magnification (Fig.20). Each experiment was performed in triplicates from two different canine AT-MSCs donors.

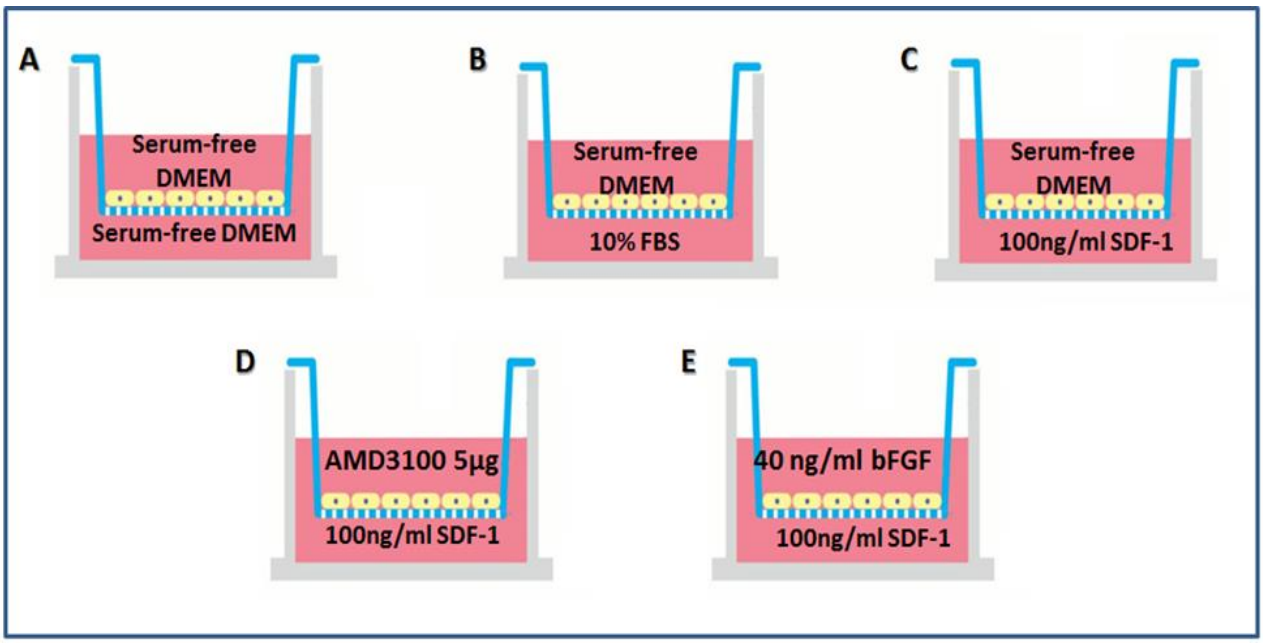

Fig 13: Experimental conditions of chemiotaxis assay

(A) Upper chamber: 1 x 10^5 cells incubated in serum-free DMEM, lower chamber: serum-free DMEM (negative control); **(B)** Upper chamber: 1 x 10^5 cells incubated in serum-free DMEM, lower chamber: 10% FBS (positive control); **(C)** Upper chamber: 1 x 10^5 cells incubated in serum free DMEM; lower chamber: 100ng/ml SDF1 (Recombinant Human/Rhesus Macaque/Feline CXCL12/SDF-1 alpha, R&D Systems); **(D)** Upper chamber: 1 x 10^5 cells pre-treated for 2h with 5µg/ml AMD3100 (Sigma) and incubated in serum free DMEM with 5µg/ml AMD3100; lower chamber: 100ng/ml SDF-1; **(E)** Upper chamber: 1 x 10^5 cells pre-treated for 24h with 40ng/ml bFGF (Miltenyi Biotec) and incubated in serum free DMEM with 40ng/ml bFGF; lower chamber: 100ng/ml SDF-1.

3.2.8. Statistical analysis

The data were analyzed using the SPSS 24IBM Software. Results are expressed as mean value ± standard deviation. The statistical significance was analyzed with the paired samples t-test and differences were considered significant for $P<0.05$.

3.3. Results

3.3.1. Isolation and phenotypic characterization of MSCs

MSCs from all the samples resulted adherent on the plastic surface 24-48h after seeding. Medium was changed every 2-3 days. Cells at P3 had the typical fibroblast-like morphology. Based on their immunophenotype they resulted to be positive for CD29, CD44 and CD90; and negative for CD34, CD45 and MHC-II (Fig.14)

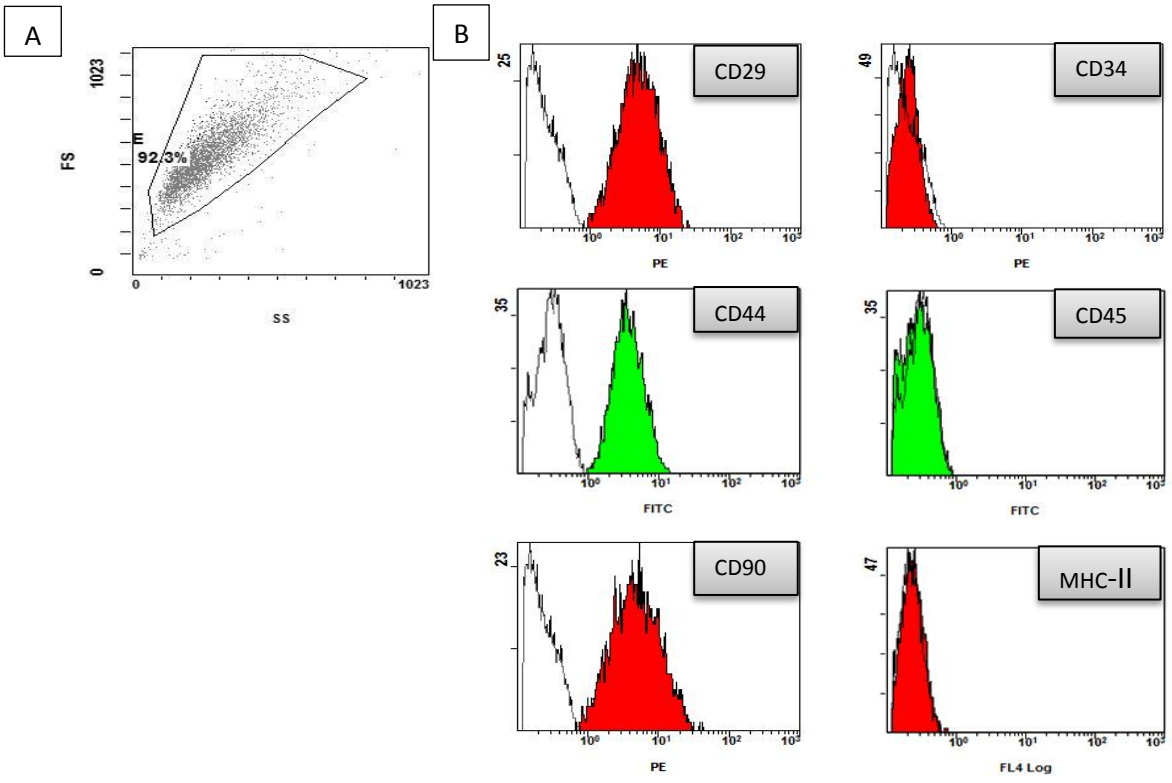

Fig 14: Representative data of the expression of cell surface markers of MSCs at P3

Live cell populations were gated in forward and side scatter for further analysis (A). Each histogram contains two peaks. The shaded represents the expression of each marker; on the other hand the transparent one is the negative control (B).

3.3.2. CXCR4 protein expression

The anti-CXCR4 antibody used in the experiment and tested on canine PBMC showed the expected reactivity. The results from the FACS analysis showed a statistically significant decrease ($P<0.05$) of the expression of the CXCR4 receptor on the surface of MSCs from P0 to P3 (Fig.15).

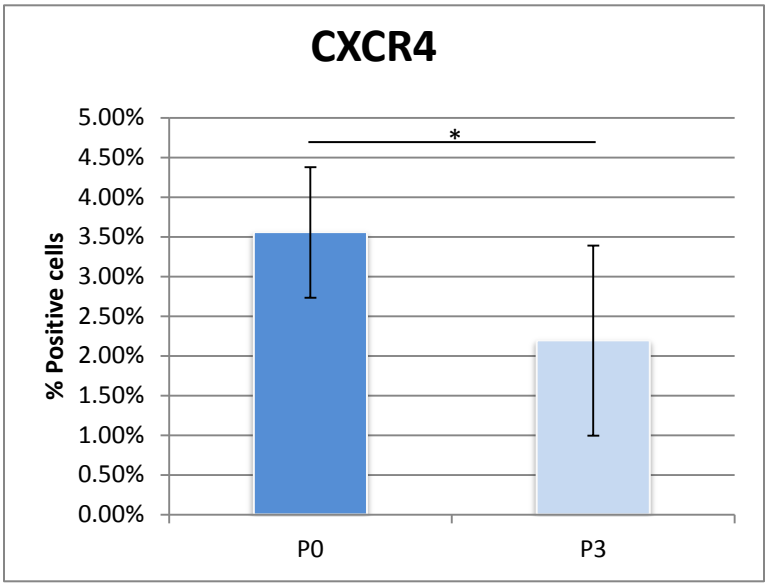

Fig 15: Percentage of CXCR4 positive cells of canine AT-MSCs analyzed at passage P0 and P3

Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of three independent experiments.

3.3.3. CXCR4 and SDF-1 gene expression

Quantitative RT-PCR analysis showed the CXCR4 and SDF-1 gene expression in MSCs at P0 and P3 (Fig. 16). The CXCR4 expression was 0.94-fold lower at P3 ($P<0.05$). Similarly, the expression of SDF-1 resulted 0.95-fold lower at P3 ($P<0.05$).

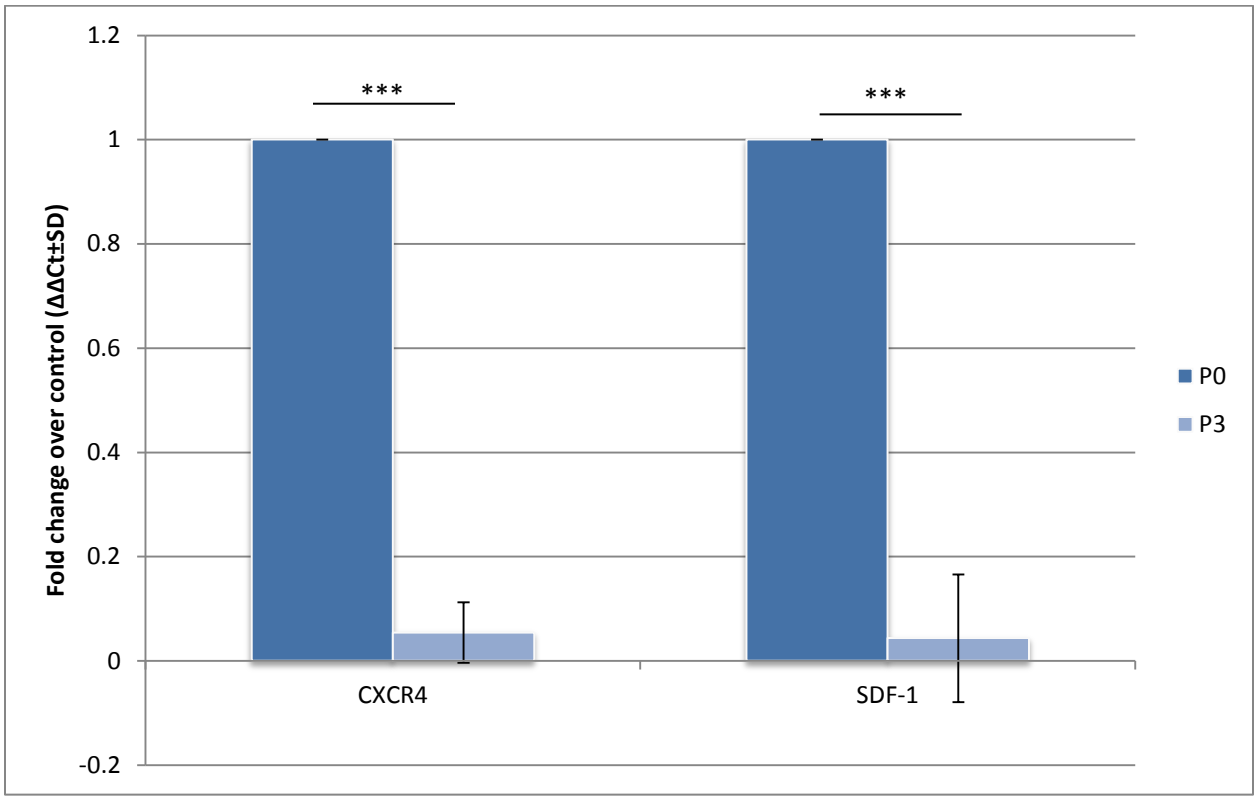

Fig 16: Expression levels of mRNAs for CXCR4 and SDF-1 in canine AT-MSCs at P0 and P3

Each value was normalized to RPS19 expression. Statistical analysis was made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates.

3.3.4. CXCR4/SDF-1α axis promotes canine MSCs migration

The chemiotaxis assays were performed to test the *in vitro* migration ability of canine AT-MSCs in response to serum, chemokines and growth factors. AT-MSCs at P3 suspended in serum free DMEM were loaded on the insert membranes and their ability to migrate was evaluated in the following conditions: (A) towards serum free DMEM; (B) towards 10% FBS; (C) towards 100ng/ml SDF-1α; (D) the cells were pretreated for 2 hours with 5μg/ml AMD3100 and during the migration assay towards 100ng/ml SDF-1α, and finally (E) the cells were pre- incubated for 24h with 40ng/ml bFGF and during the migration towards 100ng/ml SDF-1α (Fig. 17). As a negative control, one experimental condition consisted of migration of the cells towards serum-free DMEM, which tested the validity of the *in vitro* migration system. As a positive control for the migration, the cells were incubated in serum free DMEM with 10% FBS in the lower chamber.

The number of migrated cells was significantly higher when chemiotactic factors were added in the lower chamber, compared to the Transwell chambers containing serum free medium ($P<0.001$) (Fig. 18). Among the different groups, the ligand of the CXCR4 receptor, SDF-1α resulted to be the strongest chemiotactic stimulus for the cells. The number of cells that migrated towards the SDF-1α gradient was significantly higher compared to the 10% FBS stimulus ($P<0.001$). The pre-incubation with AMD3100, described as antagonist of the CXCR4 receptor (Gong et al., 2014) resulted in an inhibition of the cell migration towards SDF-1α, statistically lower than the cells non-treated with AMD3100 ($P<0.001$). However the inhibition was not complete as the cell number was still higher ($P<0.001$) than the number of cells migrating towards 10%FBS. In the last experimental condition, the cells were pre-incubated with 40ng/ml

bFGF for 24h and during the migration towards SDF-1α. The migration was statistically higher compared to the FBS group (P<0.001) and statistically lower from the SDF-1 group (P<0.001) (Fig. 19).

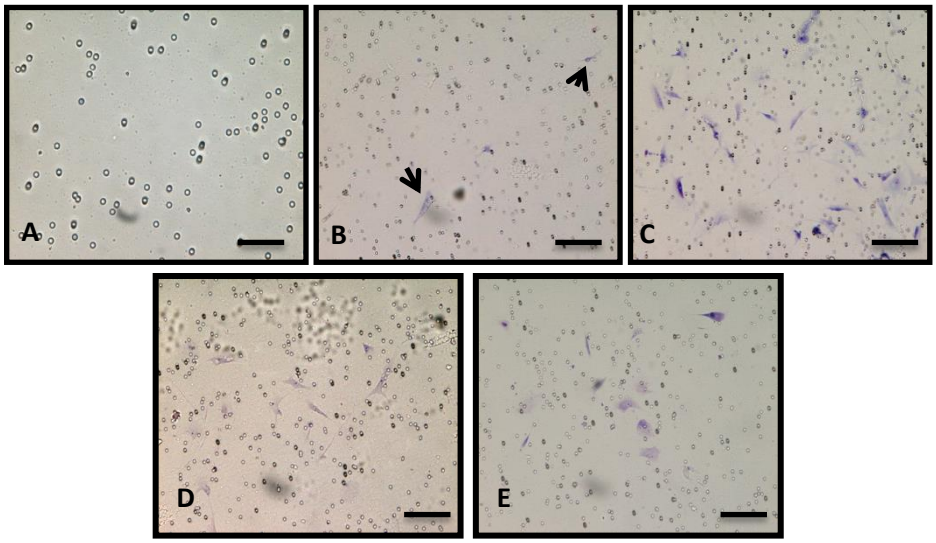

Fig 17: Representative photomicrographs of the Transwell membrane post-migration for each experimental condition

(A) = Ctrl; (B) = FBS; (C) = SDF-1α; (D) = AMD3100; (E) = bFGF;

(10x, scale bar 100μm)

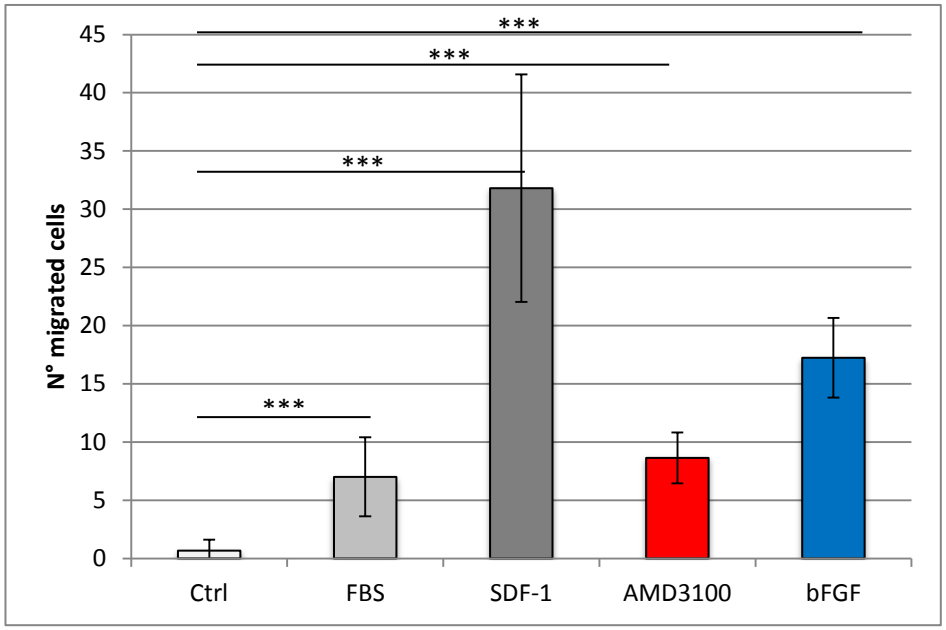

Fig 18: Number of migrated canine AT-MSCs at P3 during the in vitro chemiotaxis assay (control vs treatments)

Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates.

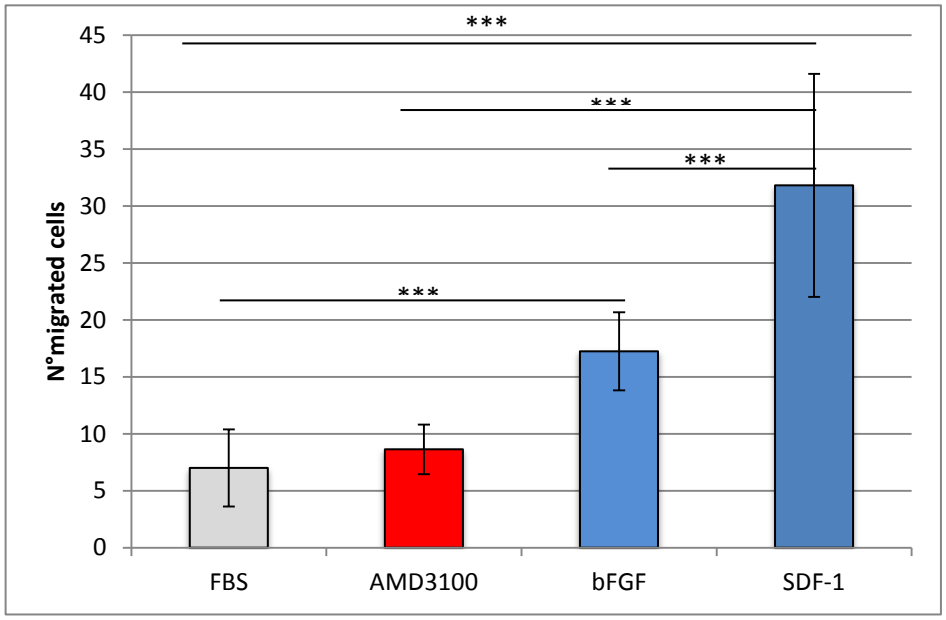

Fig 19: Number of migrated canine AT-MSCs at P3 in different chemiotactic conditions

Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates

3.4. Discussion

During the last decades, intense research activity has been dedicated to uncover the biological properties of stem cells with the final aim to understand their characteristics and use their therapeutic potential in the adequate clinical setting. In the field of veterinary medicine, MSCs have been successfully isolated from bone marrow and adipose tissue (Volk and Theoret, 2013). However, some of their biological features still need to be studied in order to understand the molecular and physiological mechanisms based on their homing and contribution to the regenerative process. One of the most attractive characteristics of MSCs is their ability to migrate and home to areas of tissue injury. For example, canine BM-MSCs injected via the systemic intravenous route homed efficiently to mandibular defects in dogs, enhanced the formation of new bone (Liu et al., 2014), and modulated the inflammation in canine patients with cruciate ligament rupture (Muir et al., 2016). Several *in vitro* and *in vivo* models in rat suggested the importance of the SDF-1α/CXCR4 axis in the trafficking of transplanted BM-MSCs (Sordi et al., 2005).

CXCR4 is a member of the chemokine receptor subfamily of seven transmembrane domain, G-protein coupled receptors, whose only known natural ligand is CXCL12/SDF-1. The roles of CXCR4 and its ligand SDF-1 in development are substantial, since knock-out mice for either protein display severe defects in the hematopoietic and nervous systems functioning and die perinatally (Ma et al., 1998). CXCR4 as a chemokine receptor is involved in leukocyte recruitment, HSCs and MSCs migration and homing, and fundamental processes such as the development of the hematopoietic, cardiovascular, and nervous systems during embryogenesis (Jacobson and Weiss, 2013). The expression of the chemokine receptor CXCR4 has been

thoroughly studied in human and murine MSCs (Wynn et al., 2004, Shi et al., 2007, Rombouts et al., 2003). Up to date, no information is available regarding the SDF-1 and CXCR4 expression in canine adipose derived MSCs. This is the first study aimed to establish the expression pattern of CXCR4 and SDF-1 in canine AT-MSCs at P0 and P3.

Canine MSCs were isolated from subcutaneous adipose tissue as a more practical cell source, and additionally the previous experiments described in Chapter 2, demonstrated the equivalence of surface markers between perivisceral and subcutaneous adipose tissue. Cells at P3 were characterized as $CD29^{+}CD44^{+}CD90^{+}CD34^{-}CD45^{-}MHCII^{-}$, in line with our previous results and published data (Takemitsu et al., 2012, Screven et al., 2014). They were cultured in complete medium and the expression of the protein CXCR4 was evaluated via FACS analysis and the gene expression via qPCR. The results of the FACS analysis demonstrated a statistically significant decrease in the surface expression of CXCR4, between the P0 and P3, although the cells at P3 did not completely lose the receptor localization on the surface. Similar data have been published for murine MSCs, where *in vitro* expansion modulates the expression of homing molecule such as chemokine receptors and adhesion molecules on murine BM-MSCs. Indeed, following *in vitro* expansion, the cells lost their homing ability (Rombouts et al., 2003). The only report available about CXCR4 expression in canine stem cells, states that at P3, 5.3% of dental pulp stem cells were positive (Iohara et al. 2011). This positivity is higher than the one reported in the present study and could be due to the different culture conditions or the fact that cell populations from different tissues have different homing abilities (Shi et al 2007)

qPCR analysis highlighted the statistically significant reduction of the mRNA expression of CXCR4 and SDF-1 in canine AT-MSCs from P0 to P3. These findings result to be in line with

the expression of the protein CXCR4 on the same cells, examined by FACS analysis. Wynn et al., studied the expression of CXCR4 in human BM-MSCs and found similar results as less than 1% of the cells resulted to be CXCR4+ by flow cytometry. Similarly, the CXCR4 mRNA expression remained generally low during the in vitro passages; however quantitative data are not available since traditional RT-PCR was used.

The *in vitro migration* of the cells was assessed with a chemotaxis assay using Transwell membrane inserts. Cells at P3 from two different donors were used for the experimental setting and each condition was performed in triplicates. Different experimental conditions were tested for the assessment of the migration ability of canine AT-MSCs. There are no published findings on the in vitro migration ability of canine MSCs. In all experimental conditions the cells were incubated in serum-free DMEM, since FBS contains chemiotactic factors that could stimulate the cells and influence their migration rate. In the first experimental condition, the cells migrated towards serum-free DMEM. Some concerns have been expressed about the validity of the *in vitro* Transwell migration system and the passage of the cells via gravity through the membrane pores. Therefore with this experimental setting, the aim was to assess the passive passage of the cells between two serum-free solutions without any chemiotactic gradient. In the second experimental setting, the migration towards 10% FBS was tested as a replicate of the standard culture conditions for MSCs. The third condition tested the migration towards DMEM containing 10%FBS and 100ng/ml SDF-1α. The concentration of the ligand of the CXCR4 receptor, SDF-1α was chosen based on previous published data in which 100ng/ml was responsible for the maximum migration effect (Gong et al., 2014). In the fourth experimental setting, the cells were pre-incubated for 2h at 37°C in serum-free DMEM and 5μg/ml AMD3100,

an antagonist of the CXCR4 receptor (Gong et al., 2014). Finally, cells were pre-incubated for 24h with serum-free DMEM and 40ng/ml bFGF before the Transwell migration assay. bFGF as a growth factor is commonly used as a supplement of MSCs culture medium due to its mitogenic activity. Additionally bFGF has been described as a modulator of the mobility of MSCs and the maximal migration was seen at the concentration of 50ng/ml (Schmidt et al., 2006b).

The in vitro analysis demonstrated that behavior of canine AT-MSCs is similar to the one of human AT-MSCs. The maximum migration rate was observed at the concentration of 100ng/ml SDF-1α. Following the incubation with AMD3100, the migration of canine AT-MSCs was significantly inhibited, which indicates the importance of the SDF-1/CXCR4 axis in the migratory ability of canine AT-MSCs. However, the inhibition was not complete and still the number of cells was statistically higher compared to the control and FBS group. This data indicated that the inhibition of CXCR4 does not completely abolish the migration of canine AT-MSCs.

The present study demonstrates that *in vitro* culture conditions induce the downregulation of the surface expression of the CXCR4 protein and the mRNA expression of CXCR4 and SDF-1 in canine AT-MSCs at P0 and P3, cultured in standard conditions. The chemiotaxis assay demonstrated the importance of the SDF-1α/CXCR4 axis in the migration of canine AT-MSCs. This finding should be taken in consideration when canine cells are amplified in vitro prior their transplantation as it can influence negatively the migration and homing of the cells, with a final low therapeutic efficacy.

Chapter 4: The synergistic effect of bFGF and hypoxic preconditioning on the modulation of CXCR4 expression in canine AT-MSCs

4.1. Background: Effects of hypoxia and bFGF on cell cultures

Cell preconditioning (CP) is an endogenous protective mechanism activated by ischemic stress, during which tissues adapt themselves by changing their cellular phenotype and function. In this way the cells become more resistant and develop tolerance to the hostile microenvironment. Therefore, CP has been used as a strategy to enhance the therapeutic potential of stem cells by exposing them for a short interval of time to a particular type of stress such as hypoxia, anoxia, acidosis, heat shock, cytokines and pharmacological treatments. The influence of pre-conditioning on the SDF-1/CXCR4 axis has been demonstrated *in vitro* and in animal models. Precisely, the benefits lie in the increment of transplanted MSCs retention and survival with the induction of a more welcoming/ favorable environment within the host tissue which leads to healing and regeneration (Cencioni et al., 2012).

Basic fibroblast growth factor (bFGF) has been reported to significantly enhance the MSCs migration, both in normoxic and hypoxic conditions. Additionally bFGF has been added at low concentrations as a supplement in culture media, since it stimulates the proliferation of the cells (Schmidt et al., 2006).

The aim of this experiment was to investigate the synergic effect of bFGF and hypoxia on the enhancement of the therapeutic action of MSCs by evaluating:

(1) The cell proliferation

(2) The expression of the CXCR4 receptor via FACS and immunohistochemistry

(3) The gene expression of CXCR4 and SDF-1 via qPCR

4.2. Materials and methods

4.2.1. Cell culture and treatments

Cryopreserved MSCs isolated from the Beagle dogs from the previous experiment were used in the present study. The viability of the cells was assessed with Trypan Blue solution (Sigma-Aldrich) before every experiment. MSCs from two different donors at P3 were seeded at a cell density of 5000 cells/cm^2 in T25 flasks. The cells were cultured in complete medium (DMEM at 37°C in humidified atmosphere in normoxic conditions (21%O_2) and hypoxic conditions (2%O_2). The day after the seeding, the cells grown in both conditions were stimulated with different concentrations of basic fibroblast growth factor (bFGF) (Miltenyi Biotec): 1ng/ml bFGF, 5ng/ml bFGF and 10ng/ml bFGF for 24h and used for further experiments.

4.2.2. MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed following the manufacturer's instructions (Sigma-Aldrich) to assess the cell proliferation. MSCs at P3 were seeded in a 96-well plate at a density of 5000 cells/cm^2 in complete growth media (DMEM + 10% FBS) at 37°C in humidified atmosphere in two different conditions: hypoxia (2% O_2) and normoxia (21%O_2). The following day, cells in both conditions were stimulated for 24h with 1ng/ml bFGF, 5ng/ml bFGF and 10 ng/ml bFGF (Miltenyi Biotec). The MTT reagent was added to the cells and incubated for 4 hours at 37°C in both conditions. The resulting formazan precipitate was solubilized with SDS 10% in HCL 0,01 N and absorbance was measured with the plate reader 1420 Multilabel Counter Victor 3 (Perkin Elmer). Each sample was analyzed in six replicates and average value was taken for plotting the graph.

4.2.3. FACS analysis

Single color flow cytometrical analysis was performed to assess the expression of CXCR4 on canine AT-MSCs at P3 in different culture conditions. As soon as the cells reached the 80% confluency, they were detached with TrypLE™ Select Enzyme (1X) (ThermoFisher Scientific) and counted with Burker's haemocytometer. For FACS analysis, 2.5 x 10^5 cells were transferred in conical and round bottom tubes, washed with 1ml sterile PBS supplemented with 1% fetal bovine serum (FBS), and finally centrifuged at 210xg for 8 min. After eliminating the supernatant, in every tube 2 µl of antibody (CD184 (CXCR4) Monoclonal Antibody (12G5), PE, eBioscience™), was added according the manufacturer's instructions. For every sample coming from the same animal, one tube containing the same number of unmarked cells was evaluated as a negative control and one sample was stained with the corresponding conjugated isotype control antibody (Mouse IgG2a kappa Isotype Control, PE, eBioscience™). Following dark incubation at 4°C for 30 min, 1ml of PBS was added and cells were centrifuged at 210xg for 8 min to remove the residual antibody. To each tube 0.5 ml of sterile PBS was added and the samples were evaluated by flow cytometry analysis (Cytomics FC 500, Beckman Coulter).

4.2.4. Immunocytochemistry

MSCs at P3 were seeded in a 4-well chamber slides (Eppendorf Cell Imaging Coverglasses Eppendorf) at a cell density of 5000 cells/cm^2 in complete growth media (DMEM + 10% FBS) at 37°C in humidified atmosphere in two different conditions: hypoxia (2% O_2) and normoxia (21%O_2). The following day, cells in both conditions were stimulated for 24h with 1ng/ml and 5ng/ml bFGF. After the treatments the culture media was eliminated, the cells were washed with PBS, fixed with acetone and freezed at -20°C until the immunocytochemistry assay. The samples

were rehydrated with PBS and washed with 3% hydrogen peroxide in 0.1% sodium azide for 10-15 min for quenching of the endogenous peroxidase. The primary antibody (Human CXCR4 Monoclonal Antibody, dilution 1:50 in 1%BSA, supplied by R&D systems) was applied for 1 h in humid chamber at room temperature. After incubation the cells were washed with PBS and then incubated for 30 min with the secondary antibodies (biotinylated sheep anti-mouse IgG, code n. RPN 1001 dilution 1:100 in PBS, supplied by Amersham Pharmacia Biotech, Little Chalfont, UK). The samples were washed and the avidin-biotin complex (VECTASTAIN® ABC Reagent, Vector Laboratories, Inc, Burlingame, USA) was added for 30 min at room temperature. Following the washing, the immunocytochemistry reaction was visualized by incubating the samples with a chromogen agent (DAB Peroxidase (HRP) Substrate, 3,3'-diaminobenzidine, Vector Laboratories, Inc, Burlingame, USA) for 2-5 minutes, until the desired staining intensity was developed. The samples were washed in distilled water, counterstained with hematoxylin for 2 minutes and washed in tap water for 5 minutes. Later they were dehydrated through 95% ethanol for 1 minute and 100% ethanol for 2x3 min. At the end they were cleared in xylene for 5 minutes and mounted with mounting medium (Eukitt®, Bio-Optica, Milano).

4.2.5. Total RNA extraction and reverse transcription (RT)

MSCs at P3 were seeded in 25cm^2 flasks (5000 cells/cm^2) in complete growth media (DMEM+10%FBS) and incubated at 37°C in humidified atmosphere in two different conditions: hypoxia (2% O_2) and normoxia (21%O_2). The following day, cells in both conditions were stimulated with 1ng/ml bFGF and 5ng/ml bFGF for 24h. Cells were trypsinized with TrypLe Select and RNA was extracted from samples of resuspended cells from each replicate culture using NucleoSpin®(Macherey-Nagel) kit following the manufacturer's instructions. The purity and concentration of the total RNA was measured by UV-spectrophotometry at 260/280 and 260nm respectively (GeneQuant Pro®, Amersham Pharmacia Biotech-GE Healthcare Life Sciences, Little Chalfont, UK). The RNA samples were stored at -80°C until retrotrascription.

Total RNA (554ng/20µl) was used for the synthesis of first strand cDNA using the High-Capacity cDNA Reverse Transciption Kit (Thermo Fisher Scientific). The reaction was performed in a PTC-100 Peltier Thermal Cycler (MJ Research) according to the manufacturer's instructions, under the following conditions: 25°C for 10 min, 37 °C for 120 min and 85°C for 5 min. The cDNA samples were stored at -20°C until qPCR analysis.

4.2.6. qPCR

The resultant cDNAs were used as templates for real-time quantitative PCR (qPCR). qPCR amplifications of C-X-C chemokine receptor type 4 (CXCR4), stromal derived factor 1 (SDF-1) and hypoxia inducible factor 1 (HIF-1 alpha) were performed. The reference gene ribosomal protein S19 (RPS19), was selected because of the stable gene expression, among other genes highly recommended for canine cells such as β-2-microglobulin (B2M), ribosomal protein S5 (RPS5), and hypoxanthine phosphoribosyltransferase (HPRT) (Penning et al., 2006). The

oligonucleotide primers used are based on published canine cDNA sequences (Iohara et al., 2011; Lee et al., 2016) or were designed (PubMed Gene) and purchased from Eurofins MWG Operon (Ebersberg, Germany). All primer pairs are positioned on different exons to reduce the chance of amplifying traces of genomic DNA (Table X). qPCR reactions were performed using 20ng cDNA and 0.3 µM of the primers in a final volume of 20 µl of 2 × concentrated SsoAdvanced™ Universal SYBR® Green Supermix (BioRad), according to the manufacturer's instructions. The cycle parameters were: 50 °C for 2 min, one denaturation step at 95 °C for 10 min and 40 repeated cycles of denaturation at 95 °C for 15 s followed by an annealing step at 60 °C for 30s and elongation at 72 °C for 30 s. At the end of the amplification cycle, a melting curve analysis for specific amplification control was performed. The gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen. Precisely, the relative gene expression of each transcript,, normalized to the RPS19 cDNA amount and expressed as relative quantities, was calculated with regards to the expression level in canine AT-MSCs at P3 cultivated in normoxia under standard culture conditions.

Gene	Accession number	Primers	Amplicon size
RPS19	XM_005616513.3	Fw: CCTTCCTCAAAAA/GTCTGGG Rv: GTTCTCATCGTAGGGAGCAAG	95bp
HIF-1α	XM_003639201	Fw: CCCAATGGATGATGACTTCC Rv: TTGGAGTCGGCTGGAATACT	113bp
CXCR4	NM_001048026.1	Fw: GGGCGAGCGGTTACCAT Rv: TGCCCA CTATGCCAGTCAAG	184bp
SDF-1	DQ182700	Fw: GCCATGAACGCCAAGGTC Rv: CTTGTTTTAGAGCTTTCTCCAGGT	270bp

Table 5: Primers used for qPCR analysis of CXCR4, SDF-1 and HIF-1α

4.2.7. Statistical analysis

The data were analyzed using the SPSS 24IBM Software. Results are expressed as mean value ± standard deviation. The statistical significance was analyzed with the paired samples t-test and differences were considered significant for $P<0.05$.

4.3. Results

4.3.1. Cell morphology

The cells in culture presented the typical fibroblast-like shape. From a morphological point of view, the supplementation of different bFGF concentrations did not affect the morphology of the cells in culture (Fig 20). The proportional effect on the proliferation was evident by the presence of a higher number of cells in .the cultures stimulated with 1ng ml and 5ng/ml bFGF both in normoxic and hypoxic conditions.

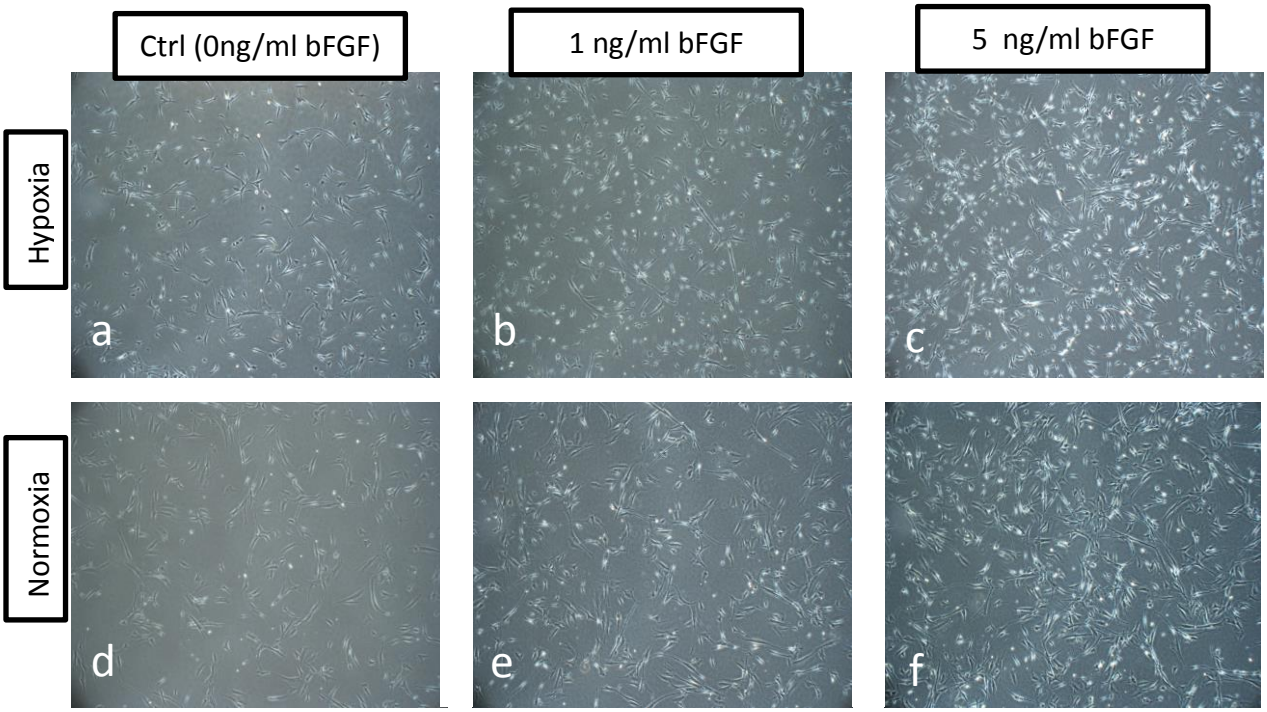

Fig 20: Photomicrographs of canine AT-MSCs

Photomicrographs of canine AT-MSCs at P3 cultured in hypoxia (a,b,c) and normoxia (d,e,f). The cells in both culture conditions were stimulated for 24h with 1ng/ml bFGF (b,e) and 5 ng/ml bFGF (c,f). Parallel control cultures were cultivated without bFGF stimulation in hypoxia (a) and normoxia (d).

4.3.2. Cell proliferation

a. Normoxia

The results of the MTT assay indicated that the addition of bFGF in the normoxic cultures stimulated the proliferation of MSCs. Compared to the control culture, there was an increase of the proliferation with the addition of 1ng/ml bFGF ($P<0.05$), 5 ng/ml bFGF and 10ng/ml bFGF ($P<0.001$). Among the different treatments, the proliferation of the cells was significantly higher in cultures supplemented with 5ng/ml bFGF ($P<0.001$) and 10ng/ml bFGF ($P<0.05$), compared to the stimulus given by 1 ng/ml bFGF (Fig.21)

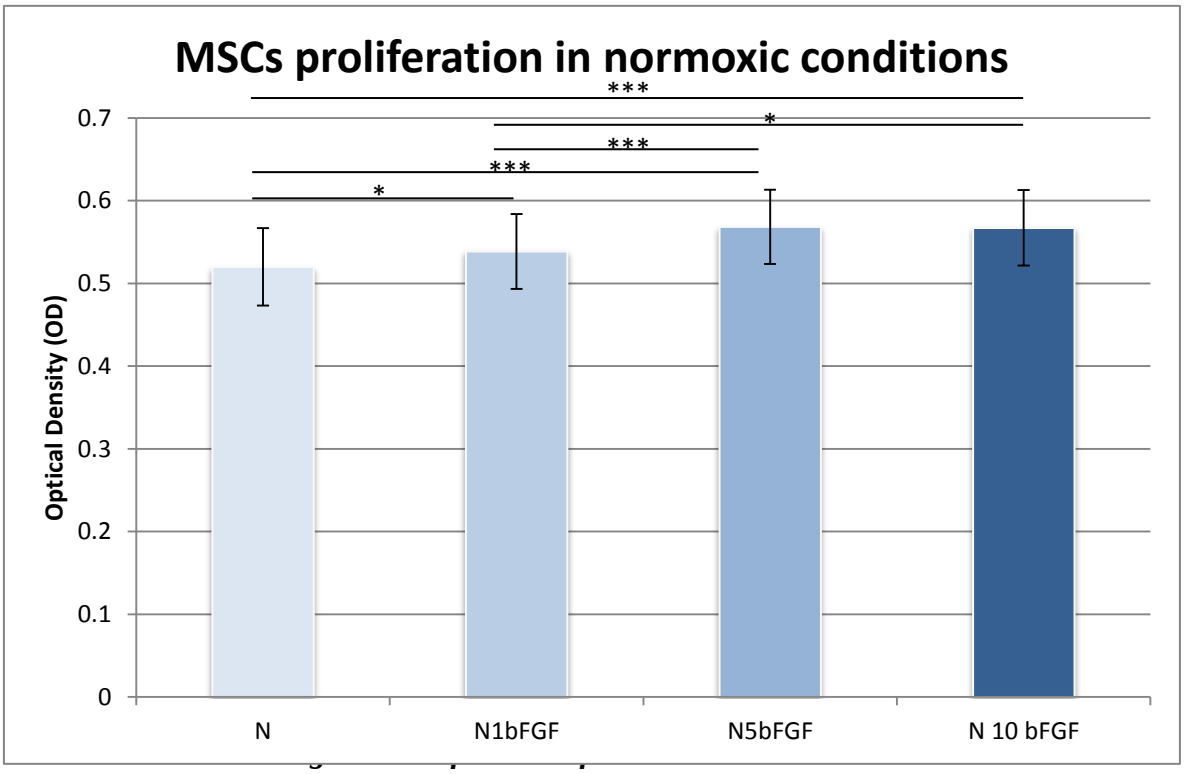

Fig 21: Proliferation of MSCs cultivated in normoxic conditions

Results are expressed as mean ±SD of two independent experiments, each performed in six replicates.

b. Hypoxia

The proliferation of the cells in hypoxic conditions resulted higher between in the treated groups with 1ng/ml bFGF (H1bFGF), 5ng/ml bFGF (H5bFGF) and 10ng/ml bFGF (H10bFGF), compared to the control group (H) ($P<0.001$). However the different concentrations of bFGF did not affect the cell proliferation, as no statistical significant differences were found among the groups (Fig. 22).

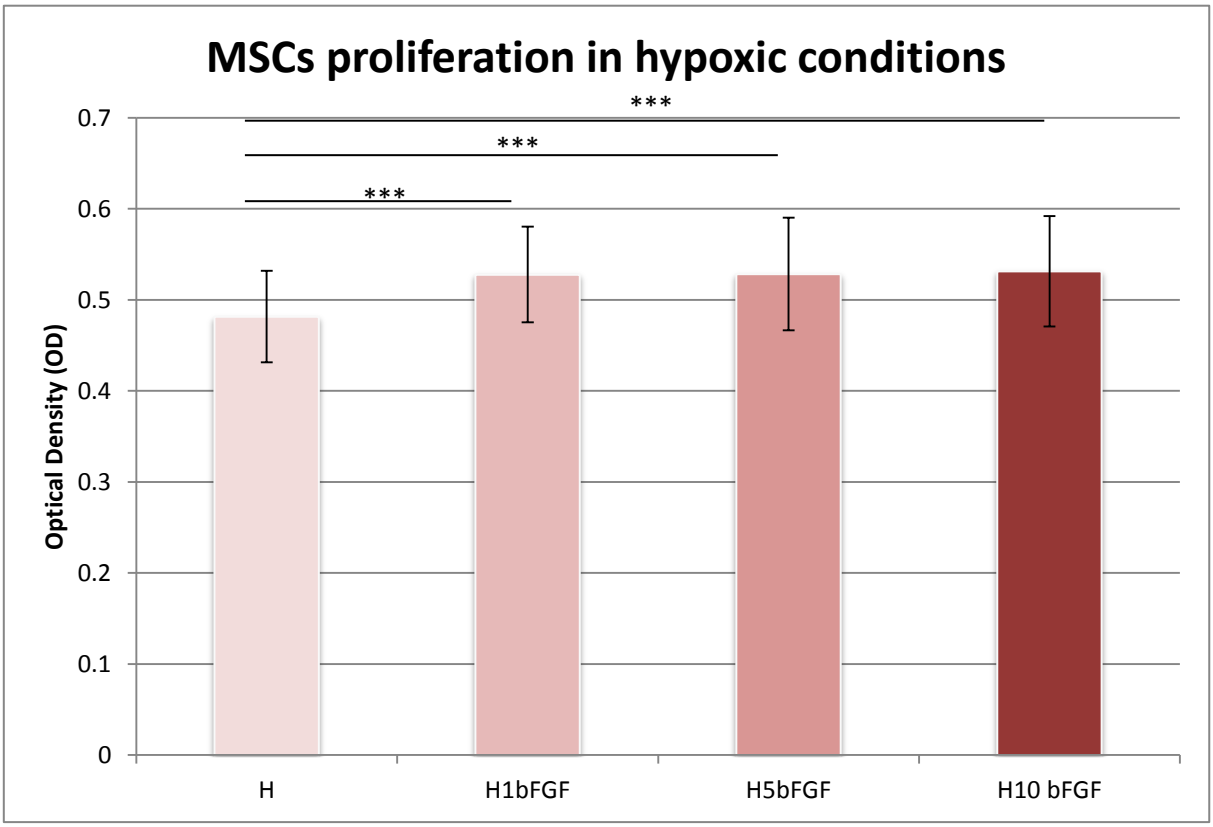

Fig 22: Proliferation of MSCs cultivated in hypoxic conditions

Results are expressed as mean ± SD of two independent experiments, each performed in six replicates.

c. Normoxia vs. hypoxia

Differences in cell proliferation where found between the cell cultures stimulated respectively with 1ng/ml bFGF, 5ng/ml bFGF and 10ng/ml bFGF grown paralelly in hypoxic and normoxic conditions. Overall, the proliferation of the cells was higher in normoxic conditions. Precisely, the cells grown without any bFGF stimulation had a higher proliferation rate in normoxic conditions ($P<0.001$). The addition of 1ng/ml bFGF resulted in higher proliferation of cells grown in normoxic conditions, although the results were not statistically significant. Cells from cultures stimulated with 5ng/ml bFGF ($P<0.001$) and 10ng/ml bFGF ($P<0.05$), had a higher proliferation rate in normoxic conditions (Fig 23).

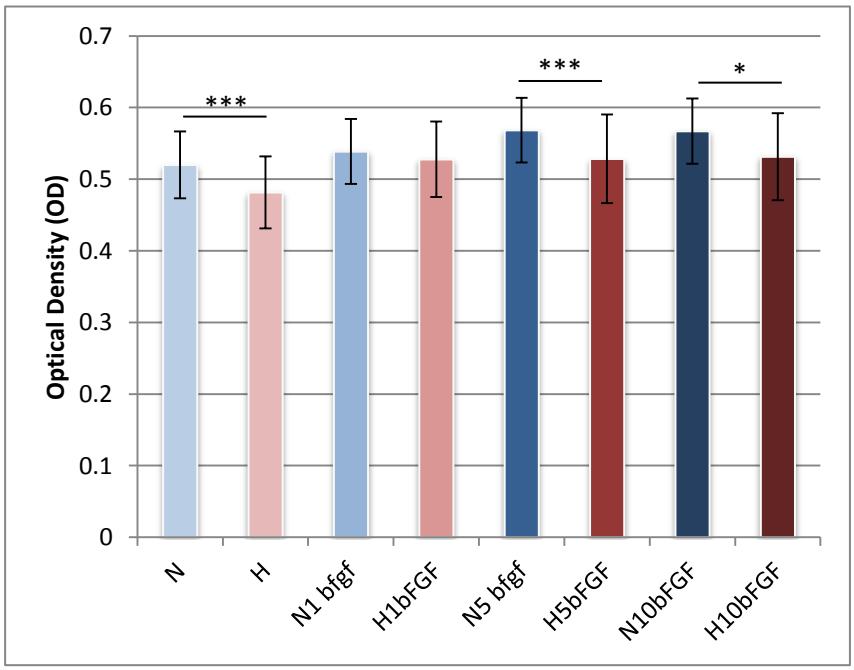

Fig 23: Proliferation of MSCs cultivated in hypoxia and normoxia, stimulated with different concentrations of bFGF

Results are expressed as mean ± SD of two independent experiments, each performed in six replicates.

4.3.3. FACS analysis

The results from the FACS analysis showed a complete absence of the surface protein CXCR4 in all the control and treated groups in hypoxic and normoxic conditions (Fig.24), indicating that the treatments did not increase the cell surface expression of CXCR4.

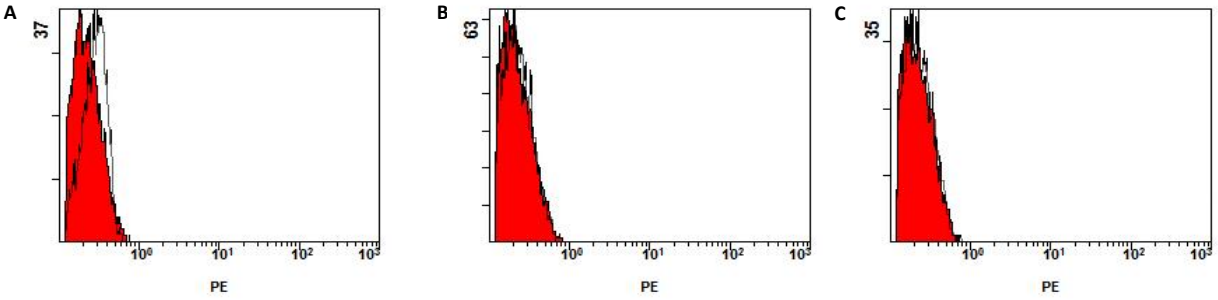

Fig 24: CXCR4 surface protein expression of MSCs stimulated with different concentrations of bFGF

Representative graphs of CXCR4 expression on MSCs cultured without bFGF (A) and treated with 1ng/ml (B) and 5ng/ml bFGF (C)

4.3.4. Immunocytochemical expression of CXCR4

The expression of the protein CXCR4 was evaluated by immunocytochemistry methods on MSCs cultivated in normoxia and hypoxia, stimulated in both conditions with 1ng/ml bFGF and 5ng/ml bFGF. Cells in all the conditions maintained the typical fibroblast-like shape. The nuclei of the cells were blue counterstained with Mayer's haematoxylin and in some of them the presence of nucleoli was evident. The control culture without primary antibody did not show any positivity. The intensity of the immunoreaction however, was different between the control and the treated groups, both in normoxic and hypoxic conditions. In all the conditions, the cells treated with 1ng/ml and 5ng/ml bFGF resulted to have a dark brown colored cytoplasm. On the contrary, the cytoplasm of the control groups was light brown. This could be related to a major expression of CXCR4 in the cytoplasm of the cells treated with bFGF. Comparing the different O2 %, in hypoxic conditions, the intensity of the staining seems to be stronger with the addition of 1ng/ml bFGF, compared to the higher concentration. On the contrary, in normoxic conditions, the staining intensity seems to be proportional to the concentration of bFGF (Fig. 25).

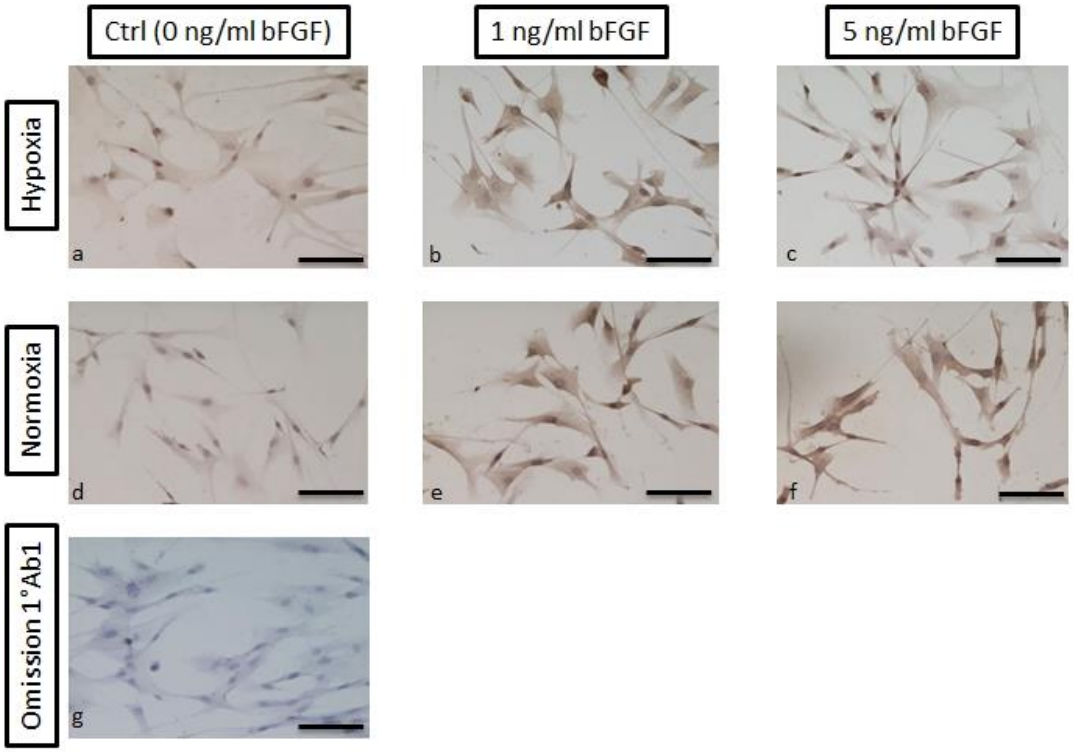

Fig 25: Immunocytochemical expression of CXCR4

Photomicrographs of canine AT-MSCs at P3 cultured in hypoxia (a,b,c) and normoxia (d,e,f,g) (10 x, scale bar 100µm). Cells in both culture conditions were stimulated for 24h with 1ng/ml bFGF (b,e) and 5 ng/ml bFGF (c,f). Parallel control cultures were cultivated without bFGF stimulation in normoxia (a) and hypoxia (d); and as a negative control for the immunohistochemistry reaction (g).

4.3.5. CXCR4 and SDF-1 mRNA expression analysis

a) CXCR4

Expression levels of canine CXCR4 were analyzed in MSCs at P3 cultivated in the following conditions: normoxia (N), normoxic conditions and supplementation of 1ng/ml bFGF (N1bFGF), normoxic conditions and supplementation of 5ng/mlbFGF (N5bFGF), hypoxia (H), hypoxic conditions and supplementation of 1ng/ml bFGF (H1bFGF) and hypoxic conditions and supplementation of 5ng/ml bFGF). The expression of CXCR4 was significantly higher in the treated groups compared to the control group (N). Precisely, CXCR4 expression was 1.8-fold higher for the N1bFGF group ($P<0.05$), 2.6-fold higher for the N5bFGF group ($P<0.01$), 0.9-fold higher for the H group ($P<0.05$), 3.1-fold higher for the H1bFGF group ($P<0.05$), and 2.5-fold higher for the H5bFGF group ($P<0.001$). Among the treated cells, statistically significant differences were found between the H and H5bFGF group, as the CXCR4 expression was 2-fold higher ($P<0.001$), and between the N1bFGF and N5bFGF group with a 1.8-fold higher expression ($P<0.05$) of CXCR4.

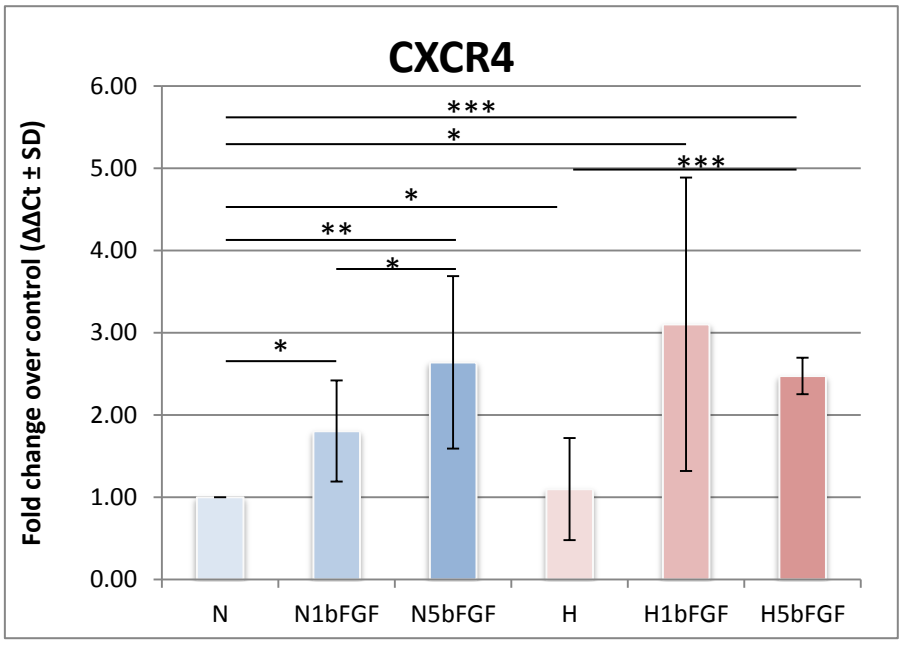

Fig 26: mRNA expression levels of mRNAs for CXCR4 in MSCs at P3 cultivated in hypoxia and normoxia with the supplementation of 1ng/ml bFGF and 5ng/ml bFGF.

Each value was normalized to ribosomal protein S19 expression. Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates.

b) SDF-1

Expression levels of SDF-1 were analyzed in MSCs at P3 cultivated in normoxia (N), normoxic conditions with supplementation of 1ng/ml bFGF (N1bFGF), normoxic conditions with supplementation of 5ng/mlbFGF (N5bFGF), hypoxia (H), hypoxic conditions and supplementation of 1ng/ml bFGF (H1bFGF) and hypoxic conditions and supplementation of 5ng/ml bFGF. In all the treated groups the expression of SDF-1 was higher compared to the control cultures. Siginificant difference in the expression of SDF-1was found among the groups N and H, with a 0.46-fold increase ($P<0.001$).

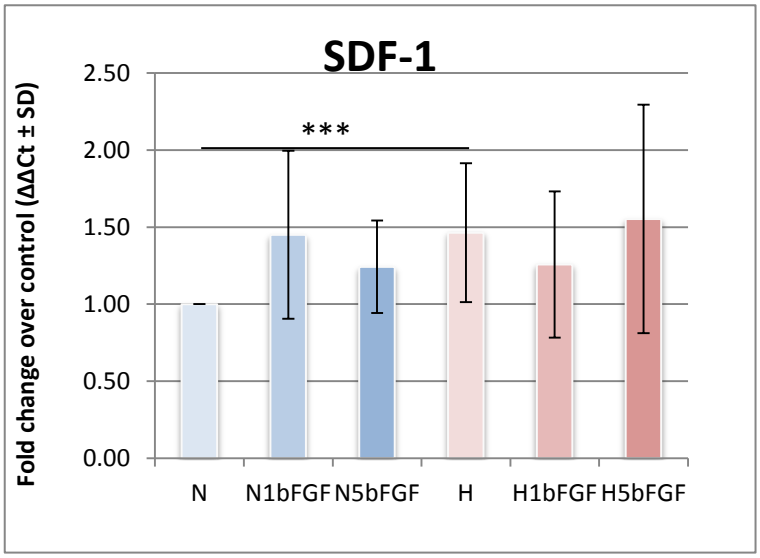

Fig 27: Expression levels of mRNAs for SDF-1 in MSCs at P3 cultivated in hypoxia and normoxia with the supplementation of 1ng/ml bFGF nd 5ng/ml bFGF.

Each value was normalized to ribosomal protein S19 expression. Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates.

c) HIF-1 α

Expression levels of canine HIF-1 α were analyzed in MSCs at P3 cultivated in the following conditions: normoxia (N), normoxic conditions and supplementation of 1ng/ml bFGF (N1bFGF), normoxic conditions and supplementation of 5ng/mlbFGF (N5bFGF), hypoxia (H), hypoxic conditions and supplementation of 1ng/ml bFGF (H1bFGF) and hypoxic conditions and supplementation of 5ng/ml bFGF. The expression of HIF-α was higher in hypoxic culture conditions compared to the normoxic cultures, although the difference did not result statistically significant. The expression in hypoxic cultures was 0.48-fold higher compared to the H5bFGF cultures ($P<0.05$) and 0.46-fold higher from the H1bFGF group ($P<0.05$). Among the normoxic cell cultures, the expression in N1bFGF was 0.54-fold higher compared to N5bFGF ($P<0.05$). Additionally, HIF-1 α was 0.44-fold higher in N1bFGF ($P<0.05$), compared to H1bFGF.

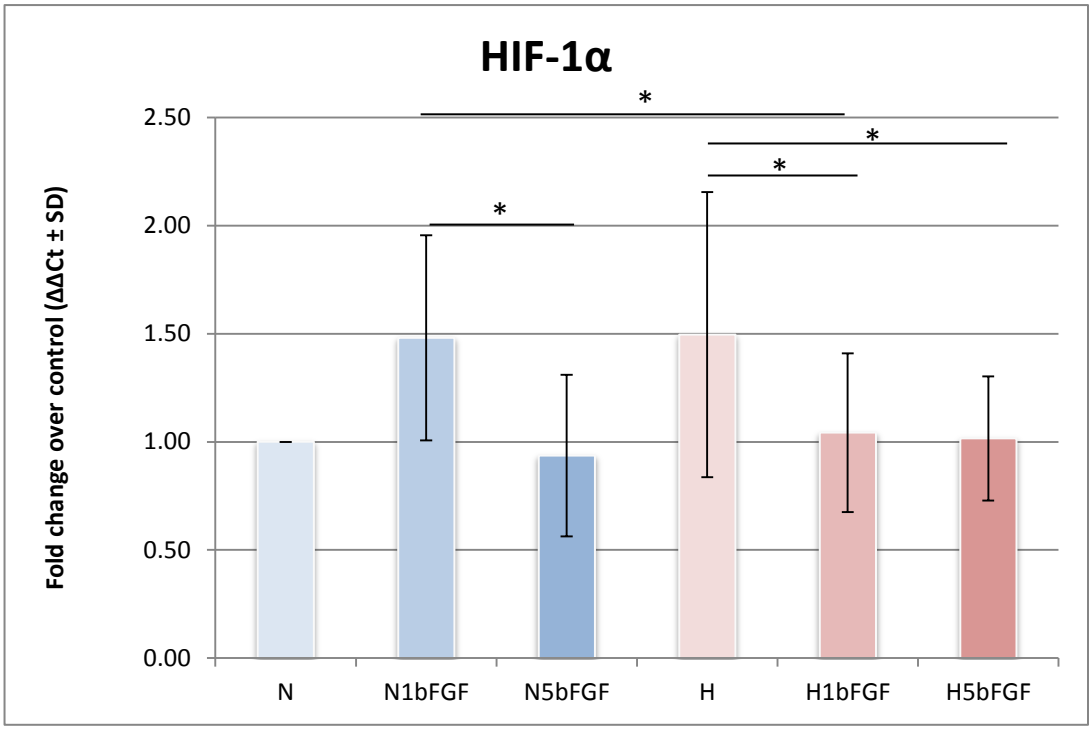

Fig. 28 ***Expression levels of mRNAs for HIF-1 α in MSCs at P3 cultivated in hypoxia and normoxia with the supplementation of 1ng/ml bFGF nd 5ng/ml bFGF.***

Each value was normalized to ribosomal protein S19 expression. Statistical comparisons were made using the paired Student's *t* test. The values are expressed as mean ± SD of two independent experiments, each performed in triplicates**.**

4.4. Discussion

The simulation of the physiological environment of the stem cell niche *in vivo,* by hypoxic preconditioning under low oxygen tension is a promising approach to optimize cell culturing. Precisely, the oxygen concentration in adipose tissue was found to be close to 3% (Ma et al., 2009). Indeed, Yamamoto et al, demonstrated the positive effect of 2%O_2 on the proliferation, multipotency, and stemness on adipose derived stem cells, compared to standard culture conditions (21%O_2) (Yamamoto et al. 2013). Additionally, the final outcome of cell-based therapies is conditioned by the engraftment of the transplanted cells and several studies have demonstrated the up-regulation of chemokine receptors (CXCR4, CXCR7, and CX3CR1) by hypoxia These receptors respond to chemokines produced by the injured tissues or organs and mediate the chemiotaxis of the transplanted MSCs to the target site (Liu et al., 2012).

On the other hand, the supplementation of cell cultures medium with growth factors has demonstrated to be an efficient method for optimizing cell proliferation. Basic fibroblast growth factor (bFGF), is part of a large family of small peptides (17-34 kDa) that stimulates cell proliferation, differentiation and migration. Due to its potent mitogen activity (which positively influences the cell growth), bFGF is commonly added as a supplement in cell cultures (Basilico C et al., 1992). Indeed, previous studies have highlighted the positive effect of bFGF on primary cultures of MSCs, as it preserves their osteogenic, adipogenic and chondrogenic potentials (Chiou et al., 2006). Schmidt et al. (2006b), described the ability of bFGF to increase the migration of human MSCs and even more interestingly, Salcedo et al., demonstrated the induction of CXCR4 gene on human endothelial cells, which promoted in vivo neovascularization (Salcedo et al., 1999).

The experiments described in Chapter 3, demonstrated the importance of the SDF1/CXCR4 axis in the migration of canine AT-MSCs and the decrease of the expression of CXCR4 and SDF-1 in canine AT-MSCs during *in vitro* amplification. The urge to optimize MSCs culture protocols in order to enhance the reduced expression of CXCR4 during extensive *in vitro* amplification, has motivated different research groups to develop methods that promote CXCR4 expression through: genetic modifications with viral (Bobis – Wozowicz et al., 2011) and non-viral vectors (Wiehe et al., 2013), pre-treatment with pro-inflammatory cytokines (Shi et al., 2007) and hypoxic preconditioning (Liu et al., 2010).

The combination of bFGF and hypoxia, was demonstrated to be an optimal method for the enhancement of the cell proliferation of human MSCs (Fábián et al., 2014). However, the synergistic effect of bFGF and hypoxic environment on the expression of CXCR4 and SDF-1 in MSCs, has not yet been assessed based on published literature.

Therefore, the goal of the present study was to evaluate the effect on CXCR4 and SDF-1 expression of a mitogen-driven *in vitro* expansion system of canine AT-MSCs, adapted to a hypoxic environment. The following parameters were evaluated: (a) cell proliferation, (b) expression of the protein CXCR4 and (c) expression of the genes CXCR4 and SDF-1, in canine AT-MSCs.

The effect on cell proliferation was assessed by MTT assay. MSCs at P3 from two donors were cultivated in normoxia (21% O_2) and hypoxia (2%O_2) and stimulated for 24 hours with different concentrations of bFGF (1 ng/ml, 5ng/ml and 10ng/ml). The concentrations of bFGF and time interval were chosen based on previous experiment that demonstrated the positive effects on the proliferation of dental bone marrow MSCs, although the results were not statistically significant

(Solenci et al. 2014). In normoxic conditions, the proliferation of the cells treated with bFGF resulted statistically higher compared to the control group. In particular, the proliferation increased proportionally with the concentrations of 1ng/ml and 5 ng/ml bFGF. The supplementation of bFGF to cell cultures in hypoxia resulted in an increase of the cell proliferation, proportionally with the concentration of bFGF, although the results were not statistically significant. Interestingly, compared to the control groups the effect of bFGF resulted statistically significant ($P<0.001$). Comparing the proliferation of cells treated with different concentrations of bFGF, grown in different oxic conditions, the normoxic ones resulted more favorable in all the groups and statistically significant for the control groups and the ones treated with 5ng/ml and 10ng/ml bFGF. Different findings have been published regarding the effect of hypoxic culture systems on canine MSCs. Lee et al. (2009), described the positive effect of hypoxia on cell proliferation and the increased stemness of the cells, in contrast with the present results.. However, Chung et al, have reported the inhibitory effect of hypoxia on canine MSCs proliferatrion and differentiation. The contradictory findings could be due to external factors such as different oxygen tensions, cell source, seeding densities, and time in culture (Chung et al., 2012).

Since the concentration of 10ng/ml bFGF did not had a higher effect on cell proliferation, in the following experiments cells were treated with 1ng/ml and 5 ng/ml bFGF. The expression of the protein CXCR4 was assessed by immunocytochemistry and flow cytometry. The results of the FACS analysis demonstrated no significant increase of the receptor on the surface of the cell treated with 1ng/ml and 5ng/ml bFGF, as the expression remained very low, similar to the basal expression at P3 in all cell cultures both in normoxia and hypoxia, treated with 1ng/ml and 5ng/ml bFGF. On the contrary, the positivity of the immunocytochemical reaction which could

be related to an elevated cytoplasmatic expression of CXCR4 seemed to be different between the control and the treated cells. In particular, in normoxic conditions the staining intensity seemed to increase proportionally with the concentrations of bFGF. However in hypoxic conditions the highest positivity was encountered at 1ng/ml bFGF. The combination of the FACS and immunocytochemical results could indicate that the preconditioning increases the cytoplasmatic protein expression of CXCR4, but possibly additional chemical, physical or environmental cues are necessary for the increase of the surface expression. Busillo and Benovic (2007), described the complex regulation of the CXCR4 signalling, in which a number of signaling molecules affect the CXCR4 expression. Additionally, Potapova et al., demonstrated that cultivating MSCs as three-dimensional aggregates restores the functional expression of CXCR4 receptor (Potapova et al., 2008).

The mRNA expression of CXCR4, SDF-1 and HIF-1 α in canine AT-MSCs, cultivated in normoxic and hypoxic conditions, stimulated with 1ng/ml and 5ng/ml bFGF was evaluated by qPCR. The hypoxic culture conditions induced a higher expression of HIF-1 α, compared to the normoxic ones, since hypoxia induces HIF-1α expression via the Erk pathway. Interestingly, the stimulation of normoxic cultures with 1ng/ml bFGF resulted in an elevated HIF-1 α expression. This effect has been described in human BM-MSCs, where bFGF induces the expression of hypoxia adaptive genes, such as HIF-1 α in normoxic cells via the ERK pathway (Fabian et al., 2014). However in the present study the supplementation of bFGF in the hypoxic cultures reduced the HIF-1 α expression. Caroti et al. reported that murine BM-MSCs cultured under 5%O_2 did not express the protein HIF-1 α, possibly due to the physiologically relevant oxygen concentration needed for the simulation of the MSCs niche. Furthermore, Caroti et al did not find an increase of Erk activation stimulated by bFGF and hypoxia. Indeed, several factors influence

the Erk pathway activation: (1) time interval of hypoxia induction, (2) level of hypoxia (1-5%O_2), (3) differences in bFGF concentrations, and (4) species differences (human vs mouse) in response to hypoxia and bFGF (Caroti et al., 2017). Therefore, in our study possibly because of these factors, the synergistic effect of bFGF and hypoxia on the Erk pathway reduced the HIF-1 α expression in canine adipose-derived MSCs in hypoxic conditions.

The CXCR4 gene expression statistically increased in all the treated groups in normoxia and hypoxia, compared to the control. In normoxia, the CXCR4 expression increased proportionally with the concentrations of bFGF, however in hypoxia the peak of CXCR4 expression corresponded to 1ng/ml bFGF. It has been demonstrated that hypoxic pre-conditioning indeed up-regulates the expression of CXCR4 (Cencioni et al., 2012, Liu et al., 2010). Additionally, Cheng et al, demonstrated the positive effect of hypoxia on CXCR4 mRNA expression in canine BM-MSCs. The effect of bFGF on CXCR4 expression was studied on human endothelial cells, where an increase of CXCR4 mRNA expression was described by Salcedo et al. The present findings are the first to demonstrate the positive effect of 1ng/ml bFGF and hypoxic pre-conditioning on CXCR4 expression on adipose derived MSCs, as it was the highest one compared to the rest of the groups.

The synergistic effect of bFGF and hypoxia increased the mRNA expression of SDF-1, although a statistically significant increase was found only between the hypoxic and normoxic cell cultures. Zagzag et al, described the positive effect of hypoxia on SDF-1α protein expression, however this is the first report of increased mRNA expression of SDF-1 α induced by hypoxic preconditioning.

The limit of the present study lies in the low number of biological samples, since it was performed on two dogs only. Although a solid conclusion cannot be made, some interesting and promising results have been obtained that can give new insights on the biology of canine adipose derived mesenchymal stem cells. The novelty of using the synergistic effect of bFGF and hypoxia seemed to enhance the cytoplasmatic expression of the protein CXCR4 and the mRNA expression of CXCR4, proportionally. This is the first study to evaluate the synergistic effect of bFGF and hypoxic preconditioning on the expression of CXCR4 of mesenchymal stem cells, and could also be used as a model for the enhancement of the expression of CXCR4 in human MSCs.

Chapter 5: Conclusions

The use of stem cells in recent decades has gained increased attention both in human and veterinary medicine. In veterinary medicine, the number of clinical studies is limited and one of the major drawbacks is represented by the lack of control groups. The promising results that have been reported, however, demonstrate the safety and efficacy of cell therapies, although the mechanisms by which they contribute to the tissue regeneration are still not fully known and understood. Therefore, there is a marked need for controlled and well-designed studies to identify the basic biologic characteristics and properties of MSCs

The goal of the present PhD thesis was to study and characterize the biologic features of canine mesenchymal stem cells with the final aim to understand and optimize their therapeutic efficacy. MSCs for therapeutic applications are classified as advanced therapy medicinal products and must be prepared according to good manufacturing practices. They require an approved and well-defined panel of assays in order to be registered for clinical use. We addressed this aspect by proposing, for the first time, a panel of species-specific canine antibodies that can be reliably used for the characterization of canine adipose-derived mesenchymal stem cells. With this panel we then characterized and compared the immunophenotype of cAT-MSCs deriving from perivisceral and subcutaneous adipose tissue. We found a substantial equivalence in the expression of surface markers, which from a practical point of view allows the veterinary surgeon to choose the source of adipose tissue sampling based on the patient and its clinical condition. Additionally, the expression of some markers was different from the parameters defined for human MSCs.

In the subsequent part of the thesis, we investigated the biological characteristics involved in the therapeutic potential of canine MSCs.

Initially, it was thought that the main effect of MSCs on tissue regeneration was provided by their ability to differentiate into other cell types. However, further studies led to a paradigm shift: the main therapeutic mechanisms of action are probably their ability to home and migrate to the injured tissue and paracrine signaling to the microenvironment and neighboring cells. The importance of the CXCR4/SDF-1α axis in the homing and migration of human and murine MSCs has been described, but no data has been published regarding the in vitro migration abilities of canine MSCs. We report the expression of the CXCR4 receptor and its ligand SDF-1 in canine adipose-derived MSCs at different *in vitro* passages, and their importance for the molecular mechanisms of cell migration. As described in human medicine, the expression was reduced during in vitro passaging. Therefore we proposed a novel in vitro cell culture system for MSCs for the enhancement of the expression of CXCR4 and SDF-1 with the final aim to improve their homing abilities. In particular, we studied the effect of bFGF and hypoxic preconditioning on MSC culture. bFGF is a known supplement in cell cultures because of its positive effect on cell proliferation. Stem cells in their native environment reside in the perivascular niche in hypoxic conditions. We demonstrated an effect on cell proliferation and an increase of CXCR4 and SDF-1 gene expression when cells were exposed to bFGF and hypoxic environment. It would be interesting to evaluate the effect on MSCs from different species and isolated from different tissues, as this system could be useful also for the optimization of culture protocols for human MSCs. The modulation of the gene expression of CXCR4 and SDF-1 are promising results, however, additional studies are necessary to understand the needed

environmental, physical and chemical cues for the increase of the surface expression of the CXCR4 protein.

Acknowledgements

- Prof. Attilio Corradi,
- Prof. Antonio Cacchioli,
- Prof. Stefano Grolli,
- Dott. Lajos Balogh

- Veterinary Anatomy, Department of Veterinary Science, Parma University, Italy
- Veterinary Biochemistry and Physiology, Department of Veterinary Science, Parma University, Italy
- National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary
- General Pathology, Department of Veterinary Science, Parma University, Italy
- Surgery Unit, Private Veterinary Clinic “Jenner”, Parma, Italy
- Surgery Unit, Private Veterinary Clinic “ San Prospero”, Reggio-Emilia, Italy

References

Ab Kadir, Ruzanna, et al. "Characterization of mononucleated human peripheral blood cells." *The Scientific World Journal* 2012 (2012).

Alamoudi, N. M., et al. "Treatment of oral ulcers in dogs using adipose tissue-derived mesenchymal stem cells." *Journal of Clinical Pediatric Dentistry* 38.3 (2014): 215-222.

Aldridge, Victoria, et al. "Human mesenchymal stem cells are recruited to injured liver in a β1-integrin and CD44 dependent manner." *Hepatology* 56.3 (2012): 1063-1073.

Allickson, Julie, and Charlie Xiang. "Human adult stem cells from menstrual blood and endometrial tissue." *Journal of Zhejiang University-Science B* 13.5 (2012): 419-420.

Andreas, Kristin, Michael Sittinger, and Jochen Ringe. "Toward in situ tissue engineering: chemokine-guided stem cell recruitment." *Trends in biotechnology* 32.9 (2014): 483-492.

Bacou, Francis, et al. "Transplantation of adipose tissue-derived stromal cells increases mass and functional capacity of damaged skeletal muscle." *Cell transplantation* 13.2 (2004): 103-111.

Baksh, D., L. Song, and R. S. Tuan. "Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy." *Journal of cellular and molecular medicine* 8.3 (2004): 301-316.

Bartsch Jr, Georg, et al. "Propagation, expansion, and multilineage differentiation of human somatic stem cells from dermal progenitors." *Stem cells and development* 14.3 (2005): 337-348.

Basilico, Claudio, and David Moscatelli. "The FGF family of growth factors and oncogenes." *Advances in cancer research* 59 (1992): 115-165.

Beer, Lucian, Michael Mildner, and Hendrik Jan Ankersmit. "Cell secretome based drug substances in regenerative medicine: when regulatory affairs meet basic science." *Annals of translational medicine* 5.7 (2017).

Binato, R., et al. "Stability of human mesenchymal stem cells during in vitro culture: considerations for cell therapy." *Cell proliferation* 46.1 (2013): 10-22.

Bjornson, Christopher RR, et al. "Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo." *Science* 283.5401 (1999): 534-537.

Black, Linda L., et al. "Effect of intraarticular injection of autologous adipose-derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs." *Veterinary therapeutics: research in applied veterinary medicine* 9.3 (2008): 192-200.

Bobis-Wozowicz, Sylwia, et al. "Genetically modified adipose tissue− derived mesenchymal stem cells overexpressing CXCR4 display increased motility, invasiveness, and homing to bone marrow of NOD/SCID mice." *Experimental hematology* 39.6 (2011): 686-696.

Bonab, Mandana Mohyeddin, et al. "Aging of mesenchymal stem cell in vitro." *BMC cell biology* 7.1 (2006): 14.

Brinkhof, Bas, et al. "Development and evaluation of canine reference genes for accurate quantification of gene expression." *Analytical biochemistry* 356.1 (2006): 36-43.

Bunnell, Bruce A., et al. "Adipose-derived stem cells: isolation, expansion and differentiation." *Methods* 45.2 (2008): 115-120.

Burchell, Jennifer T., Deborah H. Strickland, and Philip A. Stumbles. "The role of dendritic cells and regulatory T cells in the regulation of allergic asthma." *Pharmacology & therapeutics* 125.1 (2010): 1-10.

Burlacu, Alexandrina. "Tracking the mesenchymal stem cell fate after transplantation into the infarcted myocardium." *Current stem cell research & therapy* 8.4 (2013): 284-291.

Busillo, John M., and Jeffrey L. Benovic. "Regulation of CXCR4 signaling." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1768.4 (2007): 952-963.

Cargnoni, Anna, et al. "Conditioned medium from amniotic mesenchymal tissue cells reduces progression of bleomycin-induced lung fibrosis." *Cytotherapy* 14.2 (2012): 153-161.

Caroti, Courtney M., et al. "A Novel Technique for Accelerated Culture of Murine Mesenchymal Stem Cells that Allows for Sustained Multipotency." *Scientific reports* 7.1 (2017): 13334.

Caseiro, Ana Rita, et al. "Neuromuscular regeneration: perspective on the application of mesenchymal stem cells and their secretion products." *Stem cells international* 2016 (2016).

Cencioni, Chiara, Maurizio C. Capogrossi, and Monica Napolitano. "The SDF-1/CXCR4 axis in stem cell preconditioning." *Cardiovascular research* 94.3 (2012): 400-407.

Chamberlain, Giselle, et al. "Mesenchymal stem cells exhibit firm adhesion, crawling, spreading and transmigration across aortic endothelial cells: effects of chemokines and shear." *PloS one* 6.9 (2011): e25663.

Chiou, Michael, Yue Xu, and Michael T. Longaker. "Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells." *Biochemical and biophysical research communications* 343.2 (2006): 644-652.

Cho, Kyu-Sup, et al. "IFATS Collection: Immunomodulatory Effects of Adipose Tissue-Derived Stem Cells in an Allergic Rhinitis Mouse Model." *Stem Cells* 27.1 (2009): 259-265.
Chung, Dai-Jung, et al. "Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia." *Research in veterinary science* 92.1 (2012): 66-75.

Colter, David C., et al. "Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow." *Proceedings of the National Academy of Sciences* 97.7 (2000): 3213-3218.

Corcione, Anna, et al. "Human mesenchymal stem cells modulate B-cell functions." *Blood* 107.1 (2006): 367-372.

da Silva Meirelles, Lindolfo, Pedro Cesar Chagastelles, and Nance Beyer Nardi. "Mesenchymal stem cells reside in virtually all post-natal organs and tissues." *Journal of cell science* 119.11 (2006): 2204-2213.

De Becker, Ann, et al. "Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3." *Haematologica* 92.4 (2007): 440-449.

De Luca, Antonella, et al. "Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells." *Journal of cellular physiology* 226.8 (2011): 2131-2138.

Dominici, M. L. B. K., et al. "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." *Cytotherapy* 8.4 (2006): 315-317.

Dong, Feng, et al. "Myocardial CXCR4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction." *Circulation* (2012): CIRCULATIONAHA-111.

Drago, Denise, et al. "The stem cell secretome and its role in brain repair." *Biochimie* 95.12 (2013): 2271-2285.

Dragovic, Rebecca A., et al. "Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis." *Nanomedicine: Nanotechnology, Biology and Medicine* 7.6 (2011): 780-788.

Ebrahim, Neven A., and Lopa Leach. "Temporal studies into attachment, VE-cadherin perturbation, and paracellular migration of human umbilical mesenchymal stem cells across umbilical vein endothelial monolayers." *Stem cells and development* 24.4 (2014): 426-436.

Egesten, Arne, et al. "The CXC chemokine MIG/CXCL9 is important in innate immunity against Streptococcus pyogenes." *The Journal of infectious diseases* 195.5 (2007): 684-693.
Eisenberg, Leonard M., and Carol A. Eisenberg. "Stem cell plasticity, cell fusion, and transdifferentiation." *Birth Defects Research Part C: Embryo Today: Reviews* 69.3 (2003): 209-218.

English, Karen, Anna French, and Kathryn J. Wood. "Mesenchymal stromal cells: facilitators of successful transplantation?." *Cell stem cell* 7.4 (2010): 431-442.

Everaert, Bert R., et al. "Multimodal in vivo imaging reveals limited allograft survival, intrapulmonary cell trapping and minimal evidence for ischemia-directed BMSC homing." *BMC biotechnology* 12.1 (2012): 93.

Fábián, Zsolt, et al. "Basic fibroblast growth factor modifies the hypoxic response of human bone marrow stromal cells by ERK-mediated enhancement of HIF-1α activity." *Stem cell research* 12.3 (2014): 646-658.

Fierabracci, Alessandra, et al. "The use of mesenchymal stem cells for the treatment of autoimmunity: from animals models to human disease." *Current drug targets* 17.2 (2016): 229-238.

Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970;3:393–403.

Galli, Rossella, et al. "Skeletal myogenic potential of human and mouse neural stem cells." *Nature neuroscience* 3.10 (2000): 986-991.

Glennie, Sarah, et al. "Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells." *Blood* 105.7 (2005): 2821-2827

Gong, Jian, et al. "The SDF-1/CXCR4 axis regulates migration of transplanted bone marrow mesenchymal stem cells towards the pancreas in rats with acute pancreatitis." *Molecular medicine reports* 9.5 (2014): 1575-1582.

Gonzalez-Rey, Elena, et al. "Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis." *Gut* (2009).

Goodwin, Meagan, et al. "Bone marrow-derived mesenchymal stromal cells inhibit Th2-mediated allergic airways inflammation in mice." *Stem Cells* 29.7 (2011): 1137-1148.

Gregory, Carl A., et al. "How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow." *Annals of the New York Academy of Sciences* 1049.1 (2005): 97-106.

Guercio, Annalisa, et al. "Canine mesenchymal stem cells (MSCs): characterization in relation to donor age and adipose tissue-harvesting site." *Cell biology international* 37.8 (2013): 789-798.
Gupta, Naveen, et al. "Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice." *The Journal of Immunology* 179.3 (2007): 1855-1863.

Hall, M. N., et al. "Evaluation of the potential use of adipose-derived mesenchymal stromal cells in the treatment of canine atopic dermatitis: a pilot study." *Vet Ther* 11.2 (2010): 1-14.

Han, Sei-Myoung, et al. "Enhanced proliferation and differentiation of Oct4-and Sox2-overexpressing human adipose tissue mesenchymal stem cells." *Experimental & molecular medicine* 46.6 (2014): e101.

Hannafon, Bethany N., and Wei-Qun Ding. "Intercellular communication by exosome-derived microRNAs in cancer." *International journal of molecular sciences* 14.7 (2013): 14240-14269.

Honczarenko, Marek, et al. "Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors." *Stem cells* 24.4 (2006): 1030-1041.

Hou, Tianyong, et al. "Umbilical cord Wharton's Jelly: a new potential cell source of mesenchymal stromal cells for bone tissue engineering." *Tissue Engineering Part A* 15.9 (2009): 2325-2334.

Houthuijzen, J. M., et al. "The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression." *British journal of cancer* 106.12 (2012): 1901-1906.

Hsieh, Jui-Yu, et al. "Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis." *PloS one* 8.8 (2013): e72604.

Hu, Changjiang, et al. "CXCL12/CXCR4 axis promotes mesenchymal stem cell mobilization to burn wounds and contributes to wound repair." *Journal of Surgical Research* 183.1 (2013): 427-434.

Huang, GT-J., S. Gronthos, and S. Shi. "Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine." *Journal of dental research* 88.9 (2009): 792-806.

Iohara, Koichiro, et al. "Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1." *Tissue Engineering Part A* 17.15-16 (2011): 1911-1920.

Ip, James E., et al. "Mesenchymal stem cells use integrin β1 not CXC chemokine receptor 4 for myocardial migration and engraftment." *Molecular biology of the cell* 18.8 (2007): 2873-2882.

Ivanovska, Ana, et al. "Immunophenotypical characterization of canine mesenchymal stem cells from perivisceral and subcutaneous adipose tissue by a species-specific panel of antibodies." *Research in Veterinary Science* 114 (2017): 51-58.

Jacobson, Orit, and Ido Dov Weiss. "CXCR4 chemokine receptor overview: biology, pathology and applications in imaging and therapy." *Theranostics* 3.1 (2013): 1.

Jaganathan, Bithiah Grace, et al. "Rho inhibition induces migration of mesenchymal stromal cells." *Stem Cells* 25.8 (2007): 1966-1974.

Jiao, Fei, et al. "Human mesenchymal stem cells derived from limb bud can differentiate into all three embryonic germ layers lineages." *Cellular Reprogramming (Formerly" Cloning and Stem Cells")* 14.4 (2012): 324-333.

Kalra, Hina, et al. "Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation." *PLoS biology* 10.12 (2012): e1001450.

Karp, Jeffrey M., and Grace Sock Leng Teo. "Mesenchymal stem cell homing: the devil is in the details." *Cell stem cell* 4.3 (2009): 206-216.

Kassem, Moustapha, Malthe Kristiansen, and Basem M. Abdallah. "Mesenchymal stem cells: cell biology and potential use in therapy." *Basic & clinical pharmacology & toxicology* 95.5 (2004): 209-214.

Kita, Katsuhiro, et al. "Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane." *Stem cells and development* 19.4 (2010): 491-502.

Kitaori, Toshiyuki, et al. "Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model." *Arthritis & Rheumatology* 60.3 (2009): 813-823.

Kopen, Gene C., Darwin J. Prockop, and Donald G. Phinney. "Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains." *Proceedings of the National Academy of Sciences* 96.19 (1999): 10711-10716.

Krasnodembskaya, Anna, et al. "Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37." *Stem cells* 28.12 (2010): 2229-2238.

Krause, Diane S., et al. "Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell." *Cell* 105.3 (2001): 369-377.

Kumar, Sanjay, and Selvarangan Ponnazhagan. "Bone homing of mesenchymal stem cells by ectopic α4 integrin expression." *The FASEB Journal* 21.14 (2007): 3917-3927.

Lagasse, Eric, et al. "Purified hematopoietic stem cells can differentiate into hepatocytes in vivo." *Nature medicine* 6.11 (2000): 1229-1234.
Laird, Diana J., Ulrich H. von Andrian, and Amy J. Wagers. "Stem cell trafficking in tissue development, growth, and disease." *Cell* 132.4 (2008): 612-630.

Le Blanc, Katarina, et al. "Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells." *The Lancet* 363.9419 (2004): 1439-1441

Lee, Eun Young, et al. "Hypoxia-enhanced wound-healing function of adipose-derived stem cells: Increase in stem cell proliferation and up-regulation of VEGF and bFGF." *Wound Repair and Regeneration* 17.4 (2009): 540-547.

Lee, Jae-Hoon, et al. "Percutaneous transplantation of human umbilical cord blood–derived multipotent stem cells in a canine model of spinal cord injury." *Journal of Neurosurgery: Spine* 11.6 (2009): 749-757.

Lee, Jienny, et al. "Chondrogenic potential and anti-senescence effect of hypoxia on canine adipose mesenchymal stem cells." *Veterinary research communications* 40.1 (2016): 1-10.

Lee, Jin Soo, et al. "A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke." *Stem cells* 28.6 (2010): 1099-1106.

Lei, Ming, et al. "Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation." *Biomaterials* 35.24 (2014): 6332-6343.

Ley, Klaus, et al. "Getting to the site of inflammation: the leukocyte adhesion cascade updated." *Nature Reviews Immunology* 7.9 (2007): 678-689.

Li, Chun-yu, et al. "Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy." *Stem cell research & therapy* 6.1 (2015): 55.

Linge, Helena M., et al. "The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial." *Antimicrobial agents and chemotherapy* 52.7 (2008): 2599-2607.

Liu, Hongbao, et al. "Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1α in MSCs." *Biochemical and biophysical research communications* 401.4 (2010): 509-515.

Liu, Hongbao, et al. "The role of SDF-1-CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury." *PloS one* 7.4 (2012): e34608.

Ma, Qing, et al. "Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4-and SDF-1-deficient mice." *Proceedings of the National Academy of Sciences* 95.16 (1998): 9448-9453.
Ma, Teng, et al. "Hypoxia and stem cell-based engineering of mesenchymal tissues." *Biotechnology progress* 25.1 (2009): 32-42.

Manuelpillai, U., et al. "Amniotic membrane and amniotic cells: potential therapeutic tools to combat tissue inflammation and fibrosis?." *Placenta* 32 (2011): S320-S325.

Marquez-Curtis, Leah A., and Anna Janowska-Wieczorek. "Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis." *BioMed research international* 2013 (2013).

Marx, Camila, et al. "Acupoint injection of autologous stromal vascular fraction and allogeneic adipose-derived stem cells to treat hip dysplasia in dogs." *Stem cells international* 2014 (2014).

Mathivanan, Suresh, et al. "ExoCarta 2012: database of exosomal proteins, RNA and lipids." *Nucleic acids research* 40.D1 (2011): D1241-D1244.

Mei, Shirley HJ, et al. "Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis." *American journal of respiratory and critical care medicine* 182.8 (2010): 1047-1057.

Mitkari, Bhimashankar, et al. "Human bone marrow mesenchymal stem/stromal cells produce efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with cerebral ischemia, but this is not translated to behavioral recovery." *Behavioural brain research* 259 (2014): 50-59.

Moraghebi, Roksana, et al. "Term amniotic fluid: an unexploited reserve of mesenchymal stromal cells for reprogramming and potential cell therapy applications." *Stem Cell Research & Therapy* 8.1 (2017): 190

Morito, T., et al. "Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans." *Rheumatology* 47.8 (2008): 1137-1143.

Muir, Peter, et al. "Autologous bone marrow-derived mesenchymal stem cells modulate molecular markers of inflammation in dogs with cruciate ligament rupture." *PloS one* 11.8 (2016): e0159095.

Müller, Jana, et al. "Hypoxic conditions during expansion culture prime human mesenchymal stromal precursor cells for chondrogenic differentiation in three-dimensional cultures." *Cell transplantation* 20.10 (2011): 1589-1602.

Németh, Krisztián, et al. "Bone marrow stromal cells attenuate sepsis via prostaglandin E2–dependent reprogramming of host macrophages to increase their interleukin-10 production." *Nature medicine* 15.1 (2009): 42-49.
Nery, Arthur A., et al. "Human mesenchymal stem cells: from immunophenotyping by flow cytometry to clinical applications." *Cytometry Part A* 83.1 (2013): 48-61.

Neupane, Manish, et al. "Isolation and characterization of canine adipose–derived mesenchymal stem cells." *Tissue Engineering Part A* 14.6 (2008): 1007-1015.

Nitzsche, Franziska, et al. "Concise Review: MSC Adhesion Cascade—Insights into Homing and Transendothelial Migration." *STEM CELLS* 35.6 (2017): 1446-1460.

Orlic, Donald, et al. "Transplanted adult bone marrow cells repair myocardial infarcts in mice." *Annals of the New York Academy of Sciences* 938.1 (2001): 221-230.

Penha, Euler Moraes, et al. "Use of autologous mesenchymal stem cells derived from bone marrow for the treatment of naturally injured spinal cord in dogs." *Stem cells international* 2014 (2014).

Pereira, Ruth F., et al. "Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta." *Proceedings of the National Academy of Sciences* 95.3 (1998): 1142-1147.

Perez-Merino, E. M., et al. "Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: Clinical and laboratory outcomes." *The Veterinary Journal* 206.3 (2015): 385-390.

Petit, Isabelle, David Jin, and Shahin Rafii. "The SDF-1–CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis." *Trends in immunology* 28.7 (2007): 299-307.

Pevsner-Fischer, Meirav, Sarit Levin, and Dov Zipori. "The origins of mesenchymal stromal cell heterogeneity." *Stem Cell Reviews and Reports* 7.3 (2011): 560-568.

Phinney, Donald G., and Darwin J. Prockop. "Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views." *Stem cells* 25.11 (2007): 2896-2902.

Pogue, B., et al. "Stem-cell therapy for dilated cardiomyopathy: a pilot study evaluating retrograde coronary venous delivery." *Journal of Small Animal Practice* 54.7 (2013): 361-366.

Potapova, Irina A., et al. "Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells." *Journal of Biological Chemistry* 283.19 (2008): 13100-13107.

Preda, Mihai Bogdan, et al. "Remote Transplantation of Mesenchymal Stem Cells Protects the Heart Against Ischemia-Reperfusion Injury." *Stem Cells* 32.8 (2014): 2123-2134.
Puissant, Benedicte, et al. "Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells." *British journal of haematology* 129.1 (2005): 118-129.

Quesenberry, Peter J., et al. "Stem cell plasticity: an overview." *Blood Cells, Molecules, and Diseases* 32.1 (2004): 1-4.

Rafei, Moutih, et al. "Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner." *The Journal of Immunology* 182.10 (2009): 5994-6002.

Raff, Martin. "Adult stem cell plasticity: fact or artifact?." *Annual review of cell and developmental biology* 19.1 (2003): 1-22.

Ramasamy, Rajesh, et al. "Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle." *Transplantation* 83.1 (2007): 71-76.

Rani, Sweta, et al. "Mesenchymal stem cell-derived extracellular vesicles: toward cell-free therapeutic applications." *Molecular Therapy* 23.5 (2015): 812-823.

Raynaud, C. M., et al. "Comprehensive characterization of mesenchymal stem cells from human placenta and fetal membrane and their response to osteoactivin stimulation." *Stem cells international* 2012 (2012).

Reich, Christine M., et al. "Isolation, culture and chondrogenic differentiation of canine adipose tissue-and bone marrow-derived mesenchymal stem cells–a comparative study." *Veterinary research communications* 36.2 (2012): 139-148.

Riekstina, Una, et al. "Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions." *Cytotechnology* 58.3 (2008): 153-162.

Ries, Christian, et al. "MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines." *Blood* 109.9 (2007): 4055-4063.

Ringe, Jochen, et al. "Towards in situ tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2." *Journal of cellular biochemistry* 101.1 (2007): 135-146.

Rohban, Rokhsareh, and Thomas Rudolf Pieber. "Mesenchymal stem and progenitor cells in regeneration: tissue specificity and regenerative potential." *Stem cells international* 2017 (2017).

Rombouts, W. J. C., and R. E. Ploemacher. "Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture." *Leukemia* 17.1 (2003): 160-170.

Rotter, Nicole, et al. "Isolation and characterization of adult stem cells from human salivary glands." *Stem cells and development* 17.3 (2008): 509-518.

Rozemuller, Henk, et al. "Prospective isolation of mesenchymal stem cells from multiple mammalian species using cross-reacting anti-human monoclonal antibodies." *Stem Cells and Development* 19.12 (2010): 1911-1921.

Rüster, Brigitte, et al. "Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells." *Blood* 108.12 (2006): 3938-3944.

Rutenberg, Michael S., et al. "Stem cell plasticity, beyond alchemy." *International journal of hematology* 79.1 (2004): 15-21.

Saito, Yu, et al. "Homing effect of adipose-derived stem cells to the injured liver: the shift of stromal cell-derived factor 1 expressions." *Journal of hepato-biliary-pancreatic sciences* 21.12 (2014): 873-880.

Salcedo, Rosalba, et al. "Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1α." *The American journal of pathology* 154.4 (1999): 1125-1135.

Salgado, Antonio J., et al. "Mesenchymal stem cells secretome as a modulator of the neurogenic niche: basic insights and therapeutic opportunities." *Frontiers in cellular neuroscience* 9 (2015).
Sarkar, Debanjan, et al. "Engineered cell homing." *Blood* 118.25 (2011): e184-e191.

Schioppa, Tiziana, et al. "Regulation of the chemokine receptor CXCR4 by hypoxia." *Journal of Experimental Medicine* 198.9 (2003): 1391-1402.

Schmidt, Annette, et al. "Mesenchymal stem cells transmigrate over the endothelial barrier." *European journal of cell biology* 85.11 (2006): 1179-1188.

Schmidt, Annette, et al. "Basic fibroblast growth factor controls migration in human mesenchymal stem cells." *Stem cells* 24.7 (2006): 1750-1758.

Schrepfer, S., et al. "Stem cell transplantation: the lung barrier." *Transplantation proceedings*. Vol. 39. No. 2. Elsevier, 2007.

Schüring, Andreas N., et al. "Characterization of endometrial mesenchymal stem-like cells obtained by endometrial biopsy during routine diagnostics." *Fertility and sterility* 95.1 (2011): 423-426.

Screven, Rudell, et al. "Immunophenotype and gene expression profile of mesenchymal stem cells derived from canine adipose tissue and bone marrow." *Veterinary immunology and immunopathology* 161.1 (2014): 21-31.

Seifrtová, M., et al. "The response of human ectomesenchymal dental pulp stem cells to cisplatin treatment." *International endodontic journal* 45.5 (2012): 401-412

Selmani, Zohair, et al. "Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+ CD25highFOXP3+ regulatory T cells." *Stem cells* 26.1 (2008): 212-222.

Semon, Julie A., et al. "Integrin expression and integrin-mediated adhesion in vitro of human multipotent stromal cells (MSCs) to endothelial cells from various blood vessels." *Cell and tissue research* 341.1 (2010): 147-158.

Sharma, Ratti Ram, et al. "Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices." *Transfusion* 54.5 (2014): 1418-1437.

Shi, Mingxia, et al. "Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice." *Haematologica* 92.7 (2007): 897-904.

Shulman, Ziv, et al. "Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin." *Immunity* 30.3 (2009): 384-396.

Sohni, Abhishek, and Catherine M. Verfaillie. "Mesenchymal stem cells migration homing and tracking." *Stem cells international* 2013 (2013).

Sordi, Valeria, et al. "Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets." *Blood* 106.2 (2005): 419-427.

Spaggiari, Grazia Maria, et al. "Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation." *Blood* 107.4 (2006): 1484-1490.

Springer, Timothy A. "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm." *Cell* 76.2 (1994): 301-314.

Stark, Konstantin, et al. "Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and'instruct'them with pattern-recognition and motility programs." *Nature immunology* 14.1 (2013): 41-51.

Stastna, Miroslava, and Jennifer E. Van Eyk. "Secreted proteins as a fundamental source for biomarker discovery." *Proteomics* 12.4-5 (2012): 722-735.

Syková, Eva, et al. "Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury." *Cell transplantation* 15.8-1 (2006): 675-687.
Takemitsu, Hiroshi, et al. "Comparison of bone marrow and adipose tissue-derived canine mesenchymal stem cells." *BMC veterinary research* 8.1 (2012): 150.

Tang, Yao Liang, et al. "Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction." *The Annals of thoracic surgery* 80.1 (2005): 229-237.

Teo, Grace SL, et al. "Mesenchymal Stem Cells Transmigrate Between and Directly Through Tumor Necrosis Factor-α-Activated Endothelial Cells Via Both Leukocyte-Like and Novel Mechanisms." *Stem Cells* 30.11 (2012): 2472-2486.

Teo, Grace Sock Leng, et al. "Intravital imaging of mesenchymal stem cell trafficking and association with platelets and neutrophils." *Stem cells* 33.1 (2015): 265-277.

Tögel, Florian, et al. "Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury." *Kidney international* 67.5 (2005): 1772-1784.

Tuli, Richard, et al. "Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone." *Stem Cells* 21.6 (2003): 681-693.

Uccelli, Antonio, Lorenzo Moretta, and Vito Pistoia. "Mesenchymal stem cells in health and disease." *Nature reviews immunology* 8.9 (2008): 726-736.

Vanden Berg-Foels, Wendy S. "In situ tissue regeneration: chemoattractants for endogenous stem cell recruitment." *Tissue Engineering Part B: Reviews* 20.1 (2013): 28-39.

Villatoro, Antonio J., et al. "Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model." *BioMed research international* 2015 (2015).

Vizoso, Francisco J., et al. "Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine." *International journal of molecular sciences* 18.9 (2017): 1852.

Volk, Susan W., and Christine Theoret. "Translating stem cell therapies: the role of companion animals in regenerative medicine." *Wound Repair and Regeneration* 21.3 (2013): 382-394.

Walczak, Piotr, et al. "Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia." *Stroke* 39.5 (2008): 1569-1574.

Wang, Hwai-Shi, et al. "Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord." *Stem cells* 22.7 (2004): 1330-1337.

Wang, Xin, et al. "Role of mesenchymal stem cells in bone regeneration and fracture repair: a review." *International orthopaedics* 37.12 (2013): 2491-2498.
Wang, Ying, et al. "Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications." *Nature immunology* 15.11 (2014): 1009-1016.

Wei, Xin, et al. "Mesenchymal stem cells: a new trend for cell therapy." *Acta Pharmacologica Sinica* 34.6 (2013): 747-754.

Wiehe, J. M., et al. "GMP-adapted overexpression of CXCR4 in human mesenchymal stem cells for cardiac repair." *International journal of cardiology* 167.5 (2013): 2073-2081.

Wong, Rebecca SY. "Mesenchymal stem cells: angels or demons?." *BioMed Research International* 2011 (2011).

Wynn, Robert F., et al. "A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow." *Blood* 104.9 (2004): 2643-2645.

Yamamoto, Yoritsuna, et al. "Low Oxygen Tension Enhances Proliferation and Maintains Stemness of Adipose Tissue–Derived Stromal Cells." *BioResearch open access* 2.3 (2013): 199-205.

Yanez, Rosa, et al. "Adipose Tissue-Derived Mesenchymal Stem Cells Have In Vivo Immunosuppressive Properties Applicable for the Control of the Graft-Versus-Host Disease." *Stem cells* 24.11 (2006): 2582-2591.

Yilmaz, Gokhan, et al. "Selectin-mediated recruitment of bone marrow stromal cells in the postischemic cerebral microvasculature." *Stroke* 42.3 (2011): 806-811.

Ylöstalo, Joni, Nikolay Bazhanov, and Darwin J. Prockop. "Reversible commitment to differentiation by human multipotent stromal cells in single-cell–derived colonies." *Experimental hematology* 36.10 (2008): 1390-1402.

Zagzag, David, et al. "Stromal cell–derived factor-1α and CXCR4 expression in hemangioblastoma and clear cell-renal cell carcinoma: von Hippel-Lindau loss-of-function induces expression of a ligand and its receptor." *Cancer research* 65.14 (2005): 6178-6188.