



## Comparative analysis and characterization of soluble factors and exosomes from cultured adipose tissue and bone marrow mesenchymal stem cells in canine species

A.J. Villatoro<sup>a,b,1</sup>, C. Alcoholado<sup>a,c,1</sup>, M.C. Martín-Astorga<sup>a</sup>, V. Fernández<sup>a,b</sup>, M. Cifuentes<sup>a</sup>, J. Becerra<sup>a,c,d,\*</sup>

<sup>a</sup> *Laboratory of Bioengineering and Tissue Regeneration (LABRET), Department of Cell Biology, Genetics and Physiology, Faculty of Sciences, University of Málaga, IBIMA, 29071, Málaga, Spain*

<sup>b</sup> *Instituto de Immunología Clínica y Terapia Celular (IMMUNESTEM), Miraflores del Palo, 14, 29018, Málaga, Spain*

<sup>c</sup> *Networking Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), 28029, Madrid, Spain*

<sup>d</sup> *Andalusian Centre for Nanomedicine and Biotechnology-BIONAND, Severo Ochoa 35, 29590, Málaga, Spain*

### ARTICLE INFO

#### Keywords:

Cell therapy  
Mesenchymal stem cells  
Secretome  
Exosomes  
Regenerative medicine

### ABSTRACT

The two main sources of mesenchymal stem cell (MSCs) in the canine species are bone marrow (cBM-MSCs) and adipose tissue (cAd-MSCs). The secretion of multitude bioactive molecules, included under the concept of secretome and found in the cultured medium, play a predominant role in the mechanism of action of these cells on tissue regeneration. Although certain features of its characterization are well documented, their secretory profiles remain unknown. We described and compared, for the first time, the secretory profile and exosomes characterization in standard monolayer culture of MSCs from both sources of the same donor as well as its immunomodulatory potential. We found that despite the similarity in surface immunophenotyping and trilineage differentiation, there are several differences in terms of proliferation rate and secretory profile. cAd-MSCs have advantages in proliferative capacity, whereas cBM-MSCs showed a significantly higher secretory production of some soluble factors (IL-10, IL-2, IL-6, IL-8, IL-12p40, IFN- $\gamma$ , VEGF-A, NGF- $\beta$ , TGF- $\beta$ , NO and PGE2) and exosomes under the same standard culture conditions. Proteomics analysis confirm that cBM-MSCs exosomes have a greater number of characterized proteins involved in metabolic processes and in the regulation of biological processes compared to cAd-MSCs. On the other hand, secretome from both sources demonstrate similar immunomodulatory capacity when tested in mitogen stimulated lymphocyte reaction, but not in their exosomes at the dose used. Considering that the use of secretome open as a new therapeutic strategy for different diseases, without the need to implant cells, those biological differences should be considered, when choosing the MSCs source, for either cellular implantation or direct use of secretome for a specific clinical application.

### 1. Introduction

Mesenchymal stem cells (MSCs) therapy represents an important advance in the treatment of different pathologies both in human and veterinary patients. In the canine species, there are numerous studies with promising results (Villatoro et al., 2015; Villatoro et al., 2018a; Perez-Merino et al., 2015; Harman et al., 2016), however, more knowledge are needed to confirm the expectations with these new therapies (Fortier and Travis, 2011; Faltus and Brehm, 2016).

MSCs have been isolated from different tissues in canine species (Kisiel et al., 2012; Uranio et al., 2011), being bone marrow (cBM-

MSCs) and adipose tissue (cAd-MSCs) the main sources for cellular therapy in dogs, mainly because of their ease of obtaining (de Bakker et al., 2013). Both cell types share similar features such as morphology and cell surface markers, but some significant biological differences concerning their proliferation, gene expression, differentiation capacity and immune suppressive pathways were found (Chow et al., 2017; Screven et al., 2014; Russell et al., 2016).

Despite its different pleiotropic effects on tissue repair, there is an agreement that the greatest therapeutic effect of the MSCs is paracrine, through the secretion of multitude bioactive molecules included under the general concept of secretome. This secretome, is referred to a rich

\* Corresponding author.

E-mail address: [becerra@uma.es](mailto:becerra@uma.es) (J. Becerra).

<sup>1</sup> These authors contributed equally to the work

and complex mixture of different soluble factors (mainly growth factors, cytokines, chemokines and extracellular matrix components) and a variety of extracellular vesicles including exosomes, microvesicles and apoptotic bodies, and can be found in the medium where the stem cells are cultured (conditioned medium, CM) (Konala et al., 2016; Katsuda et al., 2013; Vizoso et al., 2017).

The use of secretome or any of its components open new therapeutic strategies in different diseases (Ranganath et al., 2012; Han et al., 2016; Lener et al., 2015), without needing implant cells, presenting a promising perspective to be produced as pharmaceutical products for regenerative medicine (Maguire, 2013; Pawitan, 2014; Villatoro et al., 2017). Therefore, more studies on the biological properties of the secretome and the MSCs secretory profile from different sources are necessary for a better understanding of their real therapeutic possibilities. Up to now, in veterinary patients, the paracrine profile and clinical use of MSCs secretome has been very poorly studied (Harman et al., 2017).

The objectives of this study are, firstly, to characterize cBM-MSCs and cAd-MSCs from the same donors; secondly, isolate and quantify, in the CM, soluble factors and exosomes, analyzing their proteomic profile in both types of MSCs in standard monolayer culture conditions; and third, in vitro evaluation of the immunomodulatory capacity of both MSCs, and their secretome and exosomes contents.

## 2. Materials and methods

All animal procedures were conducted by licensed veterinary surgeons. The protocols were approved by the Institutional Animal Care and Use Committee of BIONAND (Andalusian Center for Nanomedicine and Biotechnology) Málaga, Spain, and writing consent was obtained from the owners.

### 2.1. MSCs isolation

Eight different breed healthy dogs, 4 males and 4 females, aged between 1 and 3 years, and weight between 10 and 20 kg were employed as donors. Samples from inguinal fat pad and iliac crest bone marrow aspirates were obtained from the same animal under general anesthesia prior to sterilization. All animals were clinically examined previously, subjected to a routine hematological and biochemical test, being free of infectious or parasitic disease symptomatology. Any medication was administered previously at least in two months.

cAd-MSCs and cBM-MSCs were isolated and characterized as previously described by Takemitsu et al. (2012) and Villatoro et al. (2015). Adipose tissue was digested with collagenase type II (Sigma-Aldrich) and bone marrow aspirates were subjected to a density gradient on a Ficoll solution (Histopaque, Sigma-Aldrich) to separate their mononuclear fractions. Cultures was carried out in standard medium conditions: Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2.5 mM L-glutamine, 100U/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL fungizone (all from Sigma-Aldrich). Cells were trypsinised at semi-confluence and cryopreserved in liquid nitrogen. Through subsequent subcultures, our experiments were carried out on culture passage 2.

### 2.2. MSCs proliferation: population doubling time and cell proliferation

The population doubling time (PDT) was determined in both MSCs sources harvested at semi-confluence at passages 1 (p1) and 2 (p2). It was calculated using the formula  $(\log N / \log 2) / t$ , where N is the number of cells at confluence divided by the initial number of cells, and t is the number of hours in culture (Villatoro et al., 2015).

Cell proliferation was measured using MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) according to manufacturer protocol (Villatoro et al., 2015). cBM-MSCs and cAd-MSCs were seeded in a 96 well plate at a concentration of  $3 \times 10^3$  cells per well and supernatants absorbance were measured at 1, 4, 7, 11, 14,

18 and 21 days at an optical density of 490 nm using a microplate reader (ELx800, Bio-Tek instruments).

### 2.3. Immunophenotypic characterization

Fluorescence-activated cell sorting was used to characterize cBM-MSCs and cAd-MSCs at p2 as previously described by Villatoro et al. (2015) against CD29, CD44, CD73, CD90 and STRO-1 (R&D Systems), CD11/18, CD34 and CD45 (Miltenyi Biotec), and MHC-II (BD Pharmingen).

### 2.4. Multilineage differentiation

To assess the multipotentiality, MSCs were differentiated toward adipogenic, osteogenic and chondrogenic lineages using inducing mediums, according to the standard protocols previously reported by Villatoro et al. (2015). Adipogenic differentiation was evaluated by oil red O staining on days 7, 14 and 21, whereas osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity and calcium deposition with red alizarin staining on the same dates. For chondrogenic differentiation, a 3D pellet culture model was used. On day 21, pellets were subjected to routine histological processing and then stained by toluidine blue (TB), safranin O-fast green (SFG), alcian blue (AB) and immunohistochemically for type II collagen (Col-II) (all from Sigma-Aldrich).

### 2.5. Karyotype

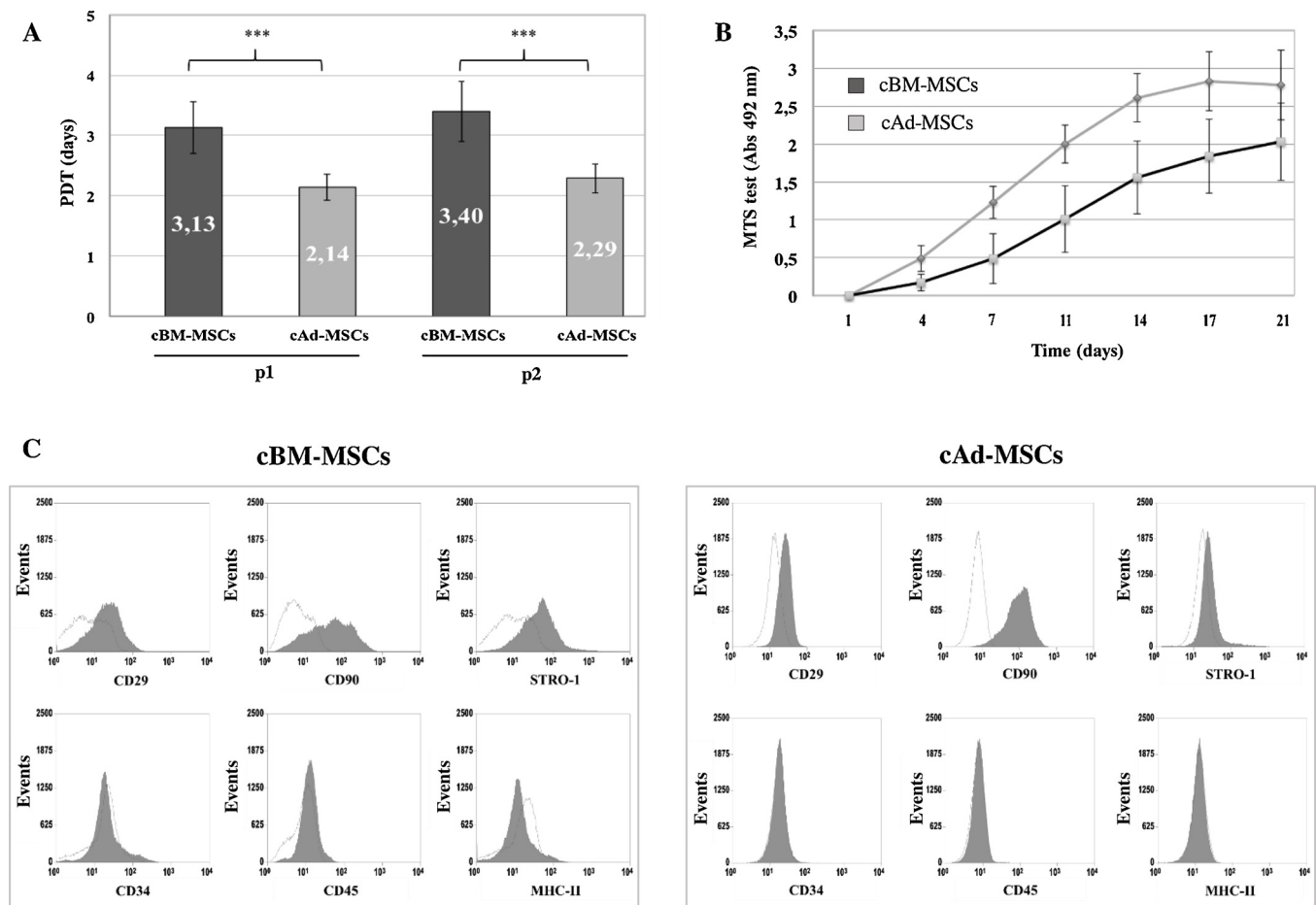
cBM-MSCs and cAd-MSCs were karyotyped as previously described by Svetlana and Stratakis, (2002), Villatoro et al. (2018b) and Calle et al. (2018). They were cultured until semi-confluence, treated with colcemid (Thermo Fisher Scientific), stained with 2% Giemsa (Merck) and analysed with ordinary bright-field microscopy.

### 2.6. CM production and quantification

cBM-MSCs and cAd-MSCs were seeded in a 96 well plate at a density of  $1.5 \times 10^6$  cells/well in standard culture medium. At semi-confluence, CM was collected, filtered (0.20 µm filter) and stored at  $-80^\circ\text{C}$ . Cell viability was evaluated with trypan blue.

Concentrations of 15 analytes were determined: chemokine (Monocyte Chemoattractant Protein-1, MCP-1); cytokines (Interleukins: IL-2, IL-6, IL-8, IL-10, IL-12p40, Tumor Necrosis Factor alpha: TNF- $\alpha$ , Interferon gamma: IFN- $\gamma$ ); immune-mediators (Prostaglandin E2: PGE2, Nitric Oxide: NO, Indoleamine 2,3-dioxygenase: IDO) and growth factors (Beta-nerve growth factor: NGF- $\beta$ , Stem Cell Factor: SCF, Transforming Growth Factor beta: TGF- $\beta$ , Vascular Endothelial growth factor A: VEGF-A). Eleven of them by the commercial Luminex kit canine cytokine 11-plex assay (Thermo Fisher Scientific), TGF- $\beta$  and PGE2 by ELISA kit (R&D) according to the manufacturers' instructions (Kornicka et al., 2018; Carrade and Borjesson, 2013; Lee et al., 2010; Kang et al., 2008).

The amounts of NO metabolites were assessed using a photometric endpoint determination method (Nitrite/Nitrate colorimetric assay kit, Roche) (Carrade and Borjesson, 2013; Kang et al., 2008). IDO enzymatic activity was measured spectrophotometrically using its metabolite, kynurenine, as previously described by Carrade et al. (2012). Briefly, 30% trichloroacetic acid (Sigma Aldrich) was added to the CM (previously supplemented with L-Tryptophan, Sigma Aldrich), incubated at  $50^\circ\text{C}$  for 30 min, and centrifuged at  $7.200\text{g}$  for 5 min. Ehrlich's reagent (Sigma Aldrich) was added to the supernatant and the optical density was measured at 490 nm (ELx800, Bio-Tek instruments). All analytes concentrations were expressed in  $\text{pg}/10^6$  cells.



**Fig. 1.** MSCs proliferation and immunophenotypic characterization. (A) Comparison of population doubling time (PDT) between cBM-MSCs (dark grey) and cAd-MSCs (light grey) in p1 and p2 with a mean  $\pm$  standard deviation (SD). Asterisks indicate significant differences between compared values  $P < 0.001$  (\*\*\*). (B) Cell metabolic activity test MTS during 21 days shows an increase of proliferative capacity of cAd-MSCs (light grey) compared to cBM-MSCs (dark grey). (C) Immunophenotype of cBM-MSCs and cAd-MSCs against some mesenchymal and hematopoietic markers with positive expression for CD29, CD90 and STRO-1 and negative for CD34, CD45 and MHC-II. Data presented as mean and standard error ( $n = 8$ ).

## 2.7. Exosomes isolation and characterization

Both type of MSCs were seeded at a density of  $1.5 \times 10^6$  cells in standard conditions until semi-confluence. Then, the cultures were washed in PBS prior to adding 20 mL of DMEM medium with 10% FBS-exosomes free.

After 3 days, CM was collected and centrifuged at 13.000 g for 30 min to remove cellular debris and microvesicles. Supernatant was centrifuged twice at 100.000 g for 60 min at 4 °C, using 70 Ti rotor in an Optima LE-80 K ultracentrifuge (Beckman Coulter). The precipitate (exosomes) was resuspended in PBS.

An exosomal fraction was placed on a carbon-Formvar-coated nickel grid (Aname), fixed and observed at different magnifications in the transmission electron microscope (TEM) (Morgagni 268D electron microscope, Philips).

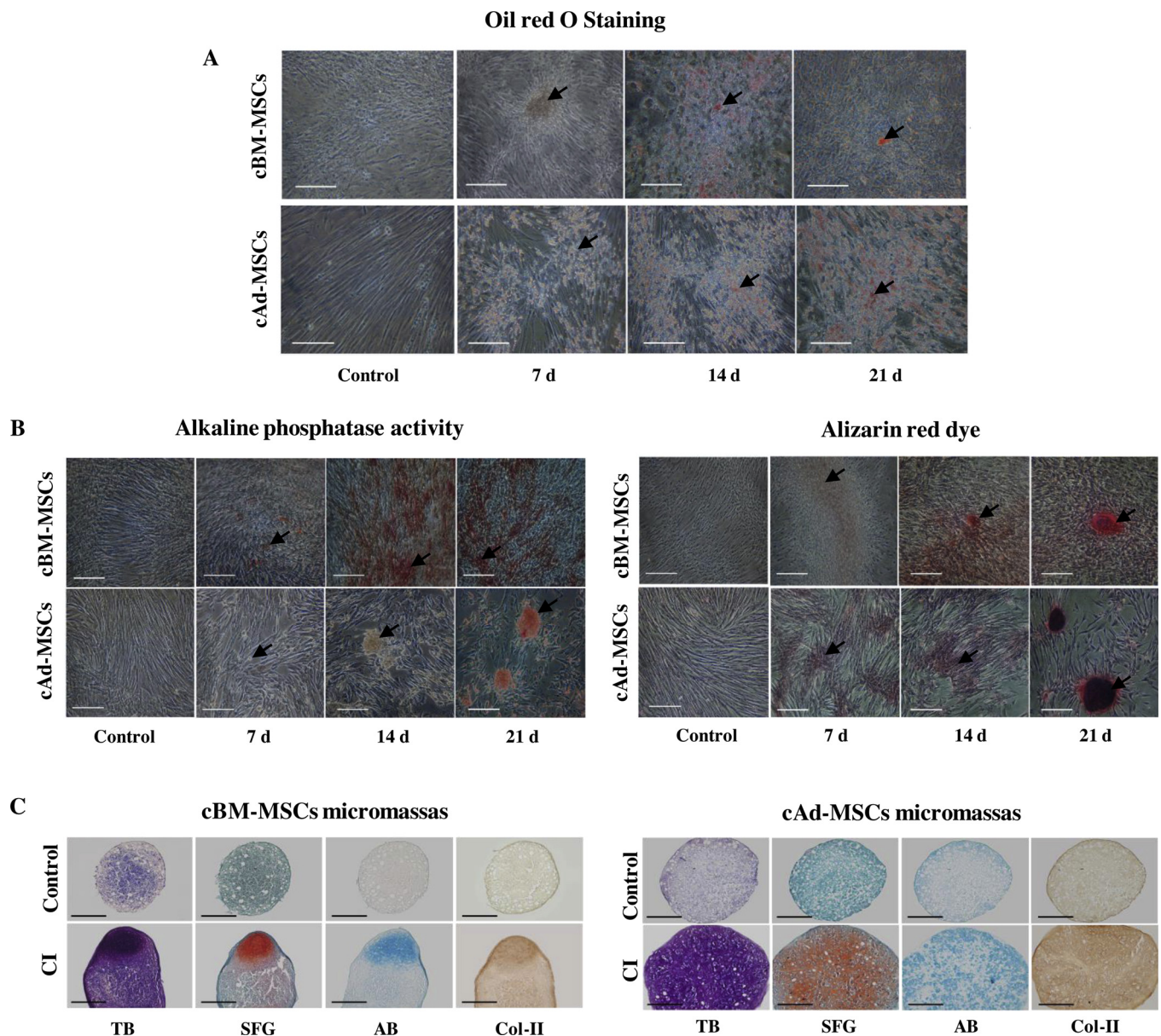
Western blot (WB) analysis was carried out with 30  $\mu$ g of cBM-MSCs and cAd-MSCs exosomes previously quantified by bicinchoninic acid (BCA) kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Proteins were separated by electrophoresis in a 10% gel SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific Scientific). After blocking, the membrane was incubated overnight at 4 °C with the mouse antibodies anti-ALIX (Abcam), anti-TSG101 (Abcam) and anti-Actin (Santa Cruz Biotechnology) and then with a secondary anti-mouse antibody (Abcam) at room temperature. Both signals were detected using enhanced chemiluminescence (ECL, GE Healthcare Life Science) and

visualized in the ChemiDoc™ XRS + system (BioRad). As a positive control, a protein lysate of the K562 cell line (BioRad) was used (Munagala et al., 2016; Lobb et al., 2015; Colombo et al., 2014; Lee et al., 2012; Mathivanan et al., 2012).

## 2.8. Exosomes proteomic analysis

To determine the exosomes proteins profile, the samples were analyzed by high resolution proteomics in accordance with the instructions provided by Central Research Support Services, SCAI at University of Málaga. The ProteinScope 3 software (Bruker Daltonics) coupled to Mascot 3.1 (Matrix Science) was used for the identification of proteins, facing the data of MS/MS against the SwissProt and NCBI nr protein sequence databases. The following parameters were taken into account: (1) Carbamidomethylation of the cysteines as a fixed modification, (2) Oxidation of methionines as a variable modification, (3) Two cut losses by trypsin, (4) Mass tolerance of 0.6 Da for precursors and 0.5 Da for fragmented masses, (5) Significant threshold: 0.05, (6) Search in the same database with inverted sequences with identical search parameters ("Peptide decoy") to estimate the number of false positives. The MS/MS spectra with a score above the threshold defined by Mascot were validated manually. The comparative study was made using Omics Comparator (Thermo Fisher Scientific) according to the biological processes provided by Gene Ontology database (Haraszi et al., 2016; Schey et al., 2015).





**Fig. 2.** *in vitro* multilineage differentiation. (A) cBM-MSCs (above) and cAd-MSCs (below) treated with Oil red O staining in order to detect red lipids droplet on days 7, 14 and 21 after adipogenic induction. Controls remain negative until 21 days. Arrows show lipid vacuoles generated after adipogenic differentiation (bars = 200  $\mu$ m) (n = 8). (B) Histochemical localization of Alkaline phosphatase activity (ALP) and Alizarin red staining of cBM-MSCs (above) and cAd-MSCs (below) at 7, 14 and 21 days. Controls remain negative until 21 days. Arrows show some of the mineralized cell aggregates (bars = 200  $\mu$ m) (n = 8). (C) Histological sections of pellets from cBM-MSCs (left) and cAd-MSCs (right) after 21 days of chondrogenesis. Maturation degree of tissues was assessed by TB, SFG, AB staining and type II collagen immunohistochemistry (bars = 500  $\mu$ m) (n = 8). Abbreviations: CI, Condro-Induced, TB, Toluidine Blue; SFG, Safranin O-Fast green; AB, Alcian Blue; Col-II, type II-collagen.

## 2.9. Immunomodulatory potential

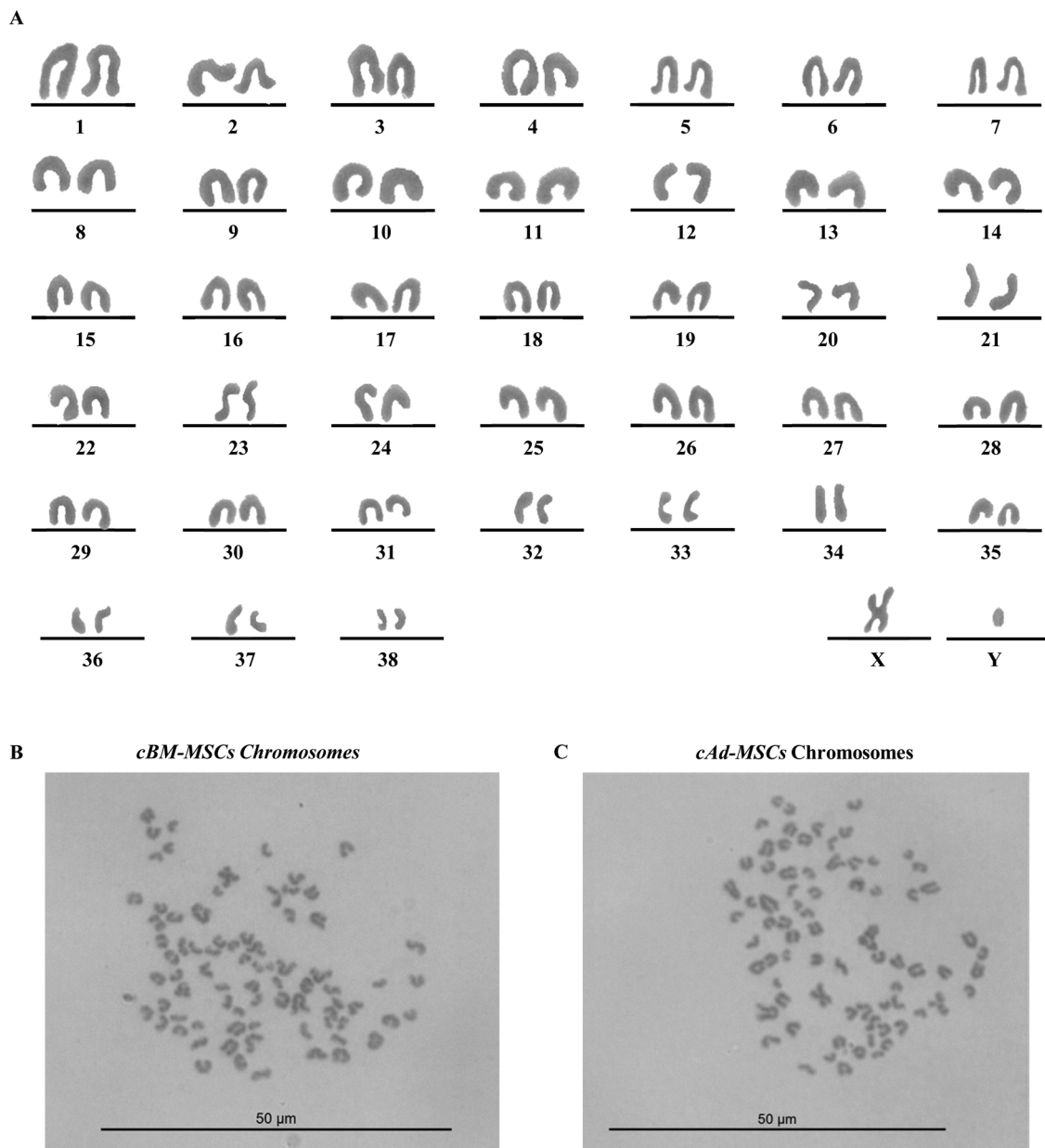
The capacity to inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) was evaluated in both MSCs, their CMs and exosomes (Toh et al., 2018; Tan et al., 2016; Kang et al., 2008; Lee et al., 2010; Kim et al., 2010). The PBMCs fraction from a healthy donor dog was separated using Ficoll-Hypaque density gradient centrifugation, stained with 4  $\mu$ M 5-chloromethylfluorescein diacetate (CMFDA, Cell Tracker Green Kit C2925, Thermo Fisher Scientific), stimulated with 5  $\mu$ g/mL ConA (Sigma Aldrich) and plated at a concentration of  $5 \times 10^4$  cells per well in a 96 well plate. The following groups were used in triplicate: PBMCs control; PBMCs and cBM/cAd-MSCs; PBMCs and cBM/cAd-MSCs-CM; PBMCs and cBM/cAd-MSCs-exosomes. According to the condition it was used  $1 \times 10^4$  cBM/cAd-MSCs

inactivated with mitomycin C (Sigma-Aldrich), 150  $\mu$ L of CM or 20  $\mu$ g/mL of exosomes from each MSCs source.

The plate was incubated for 72 h and the amount of stained PBMCs was analyzed by flow cytometer (Beckman Coulter). Data were evaluated with FlowJo cytometry software.

## 2.10. Statistical analysis

The data are presented as mean  $\pm$  standard deviation (SD). One-way analysis of variance (One-way ANOVA) was used for immunomodulatory assay and proliferation results, and the *P*-value was adjusted using the Bonferroni method for multiple comparisons. Mann-Whitney Rank Sum Test was applied for CM quantification. The degree of significance was established in the following ranges: *P* < 0.05 (\*),



**Fig. 3.** Ideogram (A) and karyotypes of cBM-MSCs (B) and cAd-MSCs (C). Ideogram represented is from cAd-MSCs, since being the same individual the ideograms are identical.

$P < 0.01$  (\*\*) and  $P < 0.001$ (\*\*\*). Data analysis was performed by SigmaPlot 11.0 software and each test was repeated three times. For PDT, MTS assay, immunophenotypic characterization and multilineage differentiation, results presented are the summarized findings of all eight animals. CM production, cytokine analysis and immunomodulatory potential were carried out in three animals of each source. Exosomes characterization and proteomic analysis were performed out using three different samples from conditioned medium of each cell type.

### 3. Results

#### 3.1. MSCs proliferation

cBM-MSCs PDT in p1 was  $3.13 \pm 0.43$  days and p2 of  $3.40 \pm 0.5$  days, without statistically significant differences between them. cAd-MSCs PDT was  $2.14 \pm 0.22$  and  $2.29 \pm 0.24$  days in p1 and p2 respectively, without significant differences between both passages. We

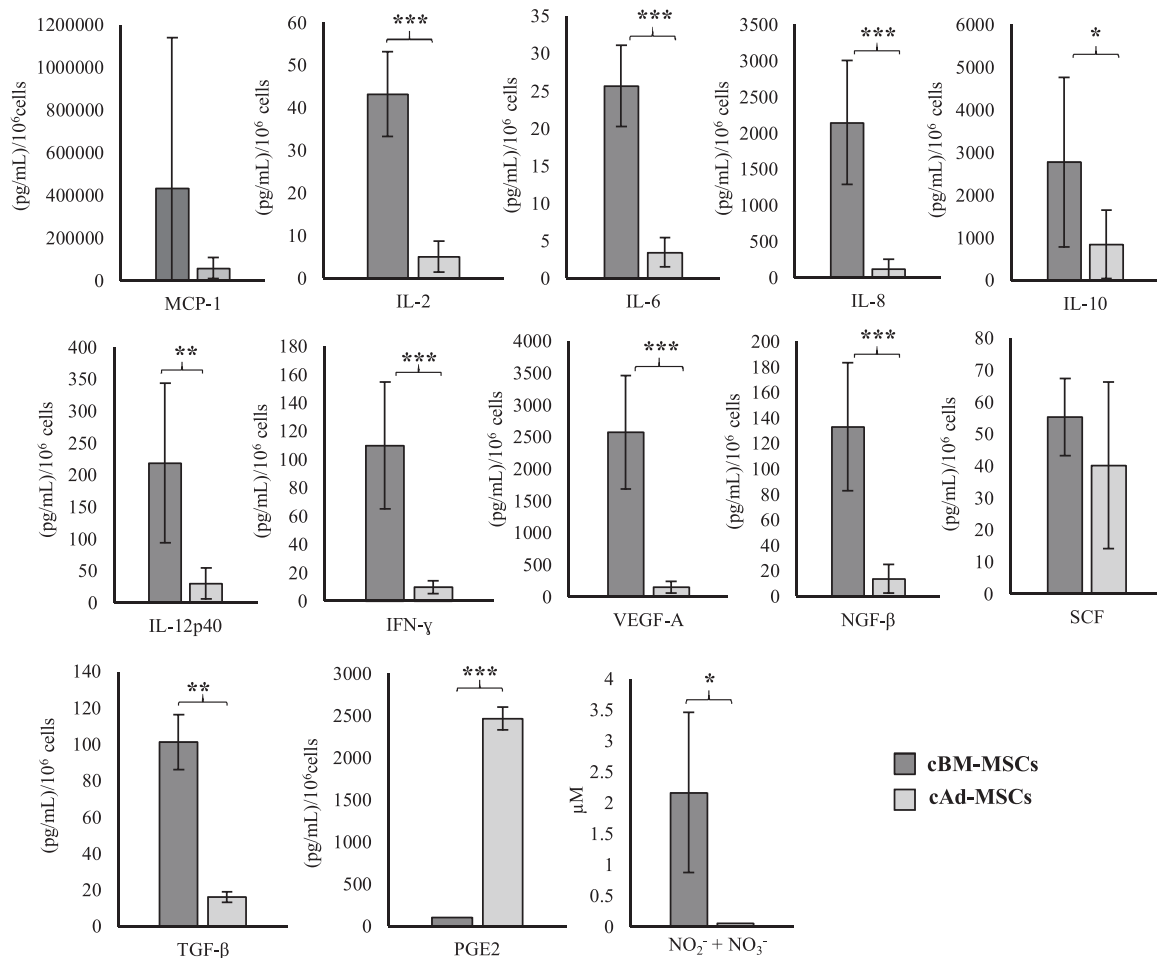
found significant differences ( $P < 0.001$ ) when comparing PDT in p1 and p2 of both sources among themselves (Fig. 1A). The cell proliferation curve during 21 days of cBM-MSCs in p2 was slower compared to cAd-MSCs (Fig. 1B).

#### 3.2. Immunophenotyping

The immunophenotypic profiles of both cell types revealed a homogenous population of cells with positive expression for the MSCs markers CD29, CD90 and STRO-1 and negative for the expression of hematopoietic markers CD34, CD45, and for MHC-II (Fig. 1C).

#### 3.3. Multilineage differentiation

Both MSCs sources differentiated into all three target phenotypes when cultured in presence of the appropriate induction medium. Adipogenic differentiation was confirmed by oil red O staining present in the cytoplasm red lipids droplet. (Fig. 2A).



**Fig. 4.** Cytokines and growth factors secretory profile of both MSCs sources (cBM-MSCs in dark grey and cAd-MSCs in light grey). Asterisks indicate significant differences between compared values  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*). Data presented as mean and standard error ( $n = 3$ ).

The osteogenic capacity was manifested by the ALP expression and the demonstration of calcium deposits positive to alizarin red staining (Fig. 2B).

For chondrogenic differentiation, the stains used confirmed the production of proteoglycans and glycosaminoglycans in the extracellular matrix, as well as the formation of collagen II (Fig. 2C).

### 3.4. Karyotype

Karyotype analyses of both MSCs at p2 were normal with diploid number of chromosomes (Fig. 3).

### 3.5. CM quantification

Secretory profile quantification in both MSCs sources is shown in Fig. 4. cBM-MSCs secreted significantly higher concentrations of IL-10, IL-2, IL-6, IL-8, IL-12p40, IFN- $\gamma$ , VEGF-A, NGF- $\beta$ , TGF- $\beta$  and NO compared to cAd-MSCs. There are not significant differences in MCP-1 and SCF. NO was only produced by cBM-MSCs and PGE2 was only secreted by cAd-MSCs, whereas IDO activity and TNF- $\alpha$  production were not observed in any case.

### 3.6. Exosomes identification

Exosome protein concentrations obtained were  $7.34 \mu\text{g} / 10^6$  of cBM-MSCs and  $0.55 \mu\text{g} / 10^6$  of cAd-MSCs (Fig. 5A). cBM-MSCs and cAd-MSCs showed positive expression of TSG101 (Tumor Susceptibility Gene 101) and ALIX markers (Fig. 5C). Presence of exosomes from both

sources were visualized by TEM (Fig. 5B).

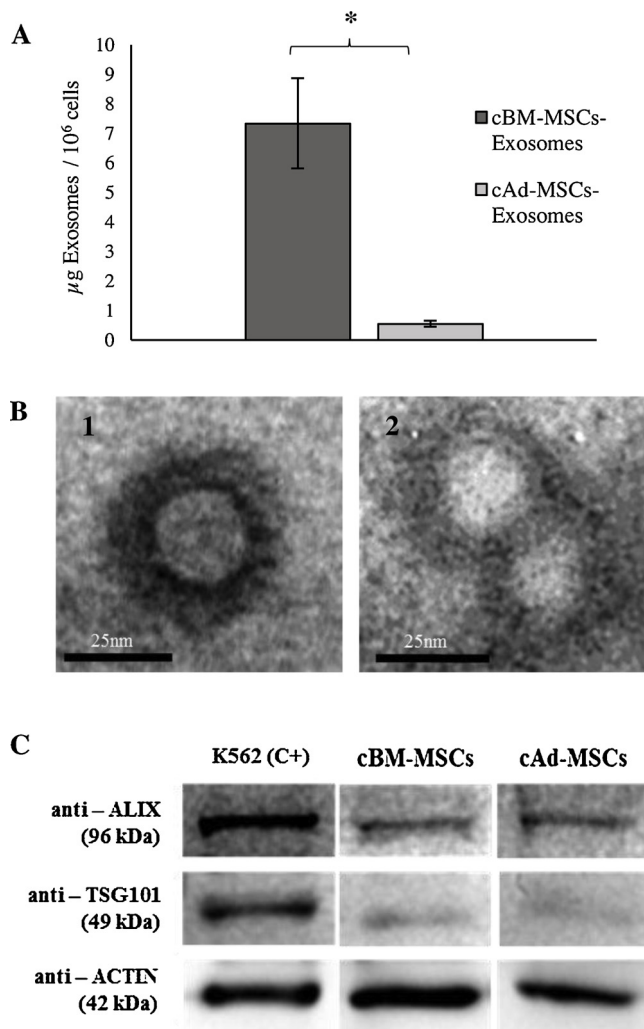
### 3.7. Proteomic analysis

The total number of peptides was performed by mass spectrometry and analyzed using Canis lupus familiaris protein database. We found 130 proteins in cBM-MSCs exosomes (77 characterized and 53 uncharacterized) and 47 proteins in cAd-MSCs exosomes (28 characterized and 19 uncharacterized). A total of 36 proteins were detected in common (Fig. 6). Biological processes of characterized exosomes proteins in both MSCs sources were determined by Gene Ontology parameters. One protein can be related with different biological functions. cBM-MSCs exosome proteins are present significantly in a major variety of physiological functions such as cell differentiation, cell organization and biogenesis, cellular component and movement, metabolic process, regulation of biological process, response to stimulus and transport compared to cAd-MSCs exosomes. Proteins involved in cell death, coagulation, conjugation and reproduction were not found. List of specific proteins involved in these different parameters are shown in tables 1, 2 and 3 at supplementary material.

### 3.8. Immunomodulatory potential

The capacity to inhibit the proliferation of PBMCs was represented in Fig. 7. Inhibitory capacity was observed for MSCs and their CMs, but without significant differences between both cell types. None of the exosomes demonstrate inhibitory capacity at the dose used.





**Fig. 5.** Characterization of cBM-MSCs and cAd-MSCs exosomes. (A) Comparative quantification of exosomes per millions of MSCs. Asterisks indicate significant differences between compared values  $P < 0.05$  (\*). (B) Representative TEM images of exosomes isolated from cBM-MSCs (B1) and cAd-MSCs (B2). Exosomes appear with a characteristic cup-shaped morphology and as round well delimited vesicles. Bars, 25 nm. (C) Western blot analysis showed a positive expression of ALIX and TSG-101 specific surface exosomal markers. Data presented as mean and standard error ( $n = 3$ ).

#### 4. Discussion

The paracrine action of MSCs plays a fundamental role in almost all the effects attributed to these cells, therefore the identification of their secreted factors is necessary to understand their therapeutic activity (Visozo et al., 2017; Lener et al., 2015). As far as we know, this is the first study comparing quantified soluble factors and exosomes secreted by cBM-MSCs and cAd-MSCs, from the same animal donors in standard monolayer culture. Our results showed that, despite the similarity in immunophenotyping and trilineage differentiation (Bearden et al., 2017; Screven et al., 2014), there are significant differences in proliferation and secretory behavior, according to the analyzed parameters. cAd-MSCs proliferation rates were significantly higher compared to cBM-MSCs (Russell et al., 2016). In both cases, they maintained a normal karyotype at the considered passage. In regenerative medicine, the limited proliferation capacity of MSCs is an important aspect to consider in order to obtain the necessary number of cells for its therapeutic use.

Regarding the secretory profile, both cells types share 12 of 15 quantified factors, but with differences in concentration. MCP-1 was the

most secreted component by both types of MSCs despite the large standard deviations shown. It is a chemokine able to attract a wide range of cells to the inflammatory niche where exert their immunomodulatory effect, in addition to promote angiogenic activity and reduce apoptosis (Kyurkchiev et al., 2014; Boomsma and Geenen, 2012). Additional studies would help to understand its contribution in the initial phases of tissue repair. All cytokines detected have a pro-inflammatory profile except IL-10, that is a pleiotropic cytokine with anti-inflammatory effect related to the induction of immune tolerance (Kyurkchiev et al., 2014). Six cytokines (IL-10, IL-2, IL-6, IL-8, IFN- $\gamma$ , IL-12p40) showed a significantly higher secretion by cBM-MSCs, highlighting IL-8, which has been shown to have potent pro-angiogenic properties and the ability to attract and activate neutrophils (Schinkothe et al., 2008).

Both CMs release different growth factors related to important biological functions as angiogenesis (VEGF-A), neurogenesis (NGF- $\beta$ ), regulation and mobilization of hematopoiesis (SCF), scarring and immunomodulation (TGF- $\beta$ ). VEGF-A, NGF- $\beta$  and TGF- $\beta$  showed significantly higher levels in cBM-MSCs-CM. The capacity of some of these factors has been evaluated in vitro in this specie (Al Delfi et al., 2016). IDO, NO and PGE2, are other notable mediators in the MSCs immunomodulatory capacity (Carrade and Borjesson, 2013). In our study, NO was only observed in cBM-MSCs, while PGE2 was only detected in cAd-MSCs. IDO activity was not showed in any case.

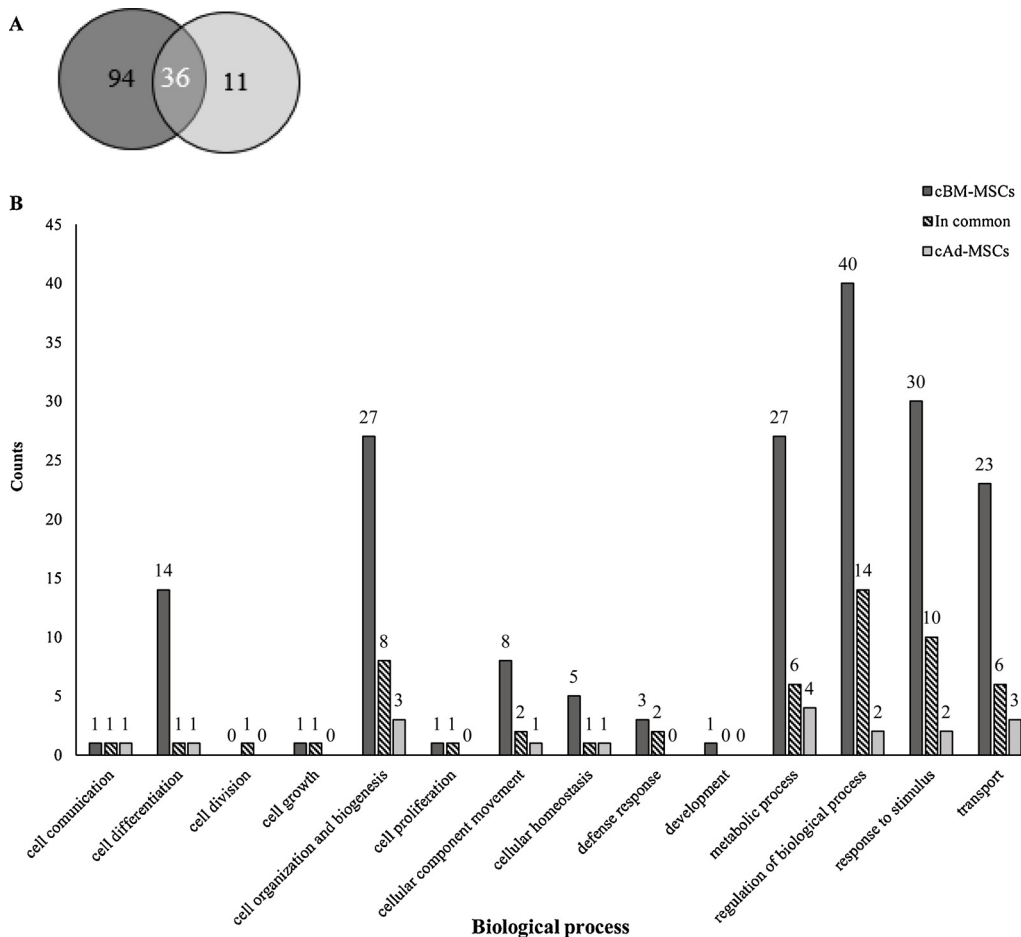
In this study, we characterized cBM-MSCs and cAd-MSCs exosomes for the first time. These have been analyzed by TEM to detect their shape and size, and by WB for the expression of specific exosomal surface protein markers such as ALIX and TSG101 (Reiner et al., 2017) involved in exosomes biogenesis mediated by endosomal sorting complexes required for transport (ESCRT). Despite similarities in size and markers expression, cBM-MSCs significantly produce more exosomes (13 times) compared to cAd-MSCs, in our experimental conditions.

In other species, exosomes containing ubiquitous common proteins and cell-type specific proteins have been reported (Blazquez et al., 2014). We have been able to identify 36 common proteins between both sources, as well as differences in the number of specific proteins (94 in cBM-MSCs exosomes and 11 in cAd-MSCs exosomes). Our proteomics results confirm the highest secretory profile of cBM-MSCs, since the exosomes from this cell type have a greater number of characterized proteins mainly involved in cell differentiation, cell organization and biogenesis, metabolic activity, regulation of biological process, response to stimulus and transport compared to cAd-MSCs. Nonetheless, more proteomics studies are necessary for the complete identification of proteins and processes and for being able to infer their therapeutic possibilities. Recently the interest of exosomes as a therapeutic element has increased thanks to their results in different disease models, highlighting the need for standardization in their production and use (Reiner et al., 2017; Lener et al., 2015). Regardless of its different secretory profile, cBM-MSCs and cAd-MSCs were roughly equivalent in terms of their ability to suppress T cell activation, similar to the published in dogs (Chow et al., 2017; Russell et al., 2016) and other species (Chae et al., 2017; Carrade and Borjesson, 2013). Likewise, both CMs showed similar immunomodulatory capacity, without differences between sources and similar to other species (Pawitan, 2014).

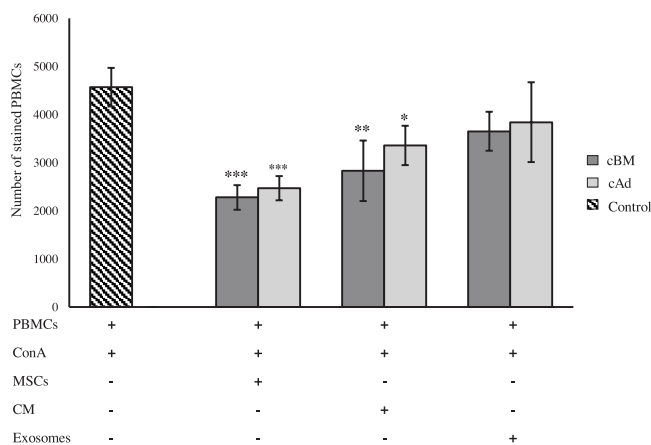
However, canine MSCs exosomes do not have suppressive effect on the PBMCs proliferation at the dose used. New studies with other concentrations would be interesting to determine the immunomodulatory effect and therapeutics possibilities.

We demonstrated that both canine MSCs-CM under standard culture conditions, contain different molecules with regenerative potential and immunomodulatory capacity, whose composition is tissue source-dependent, as it has been proposed in humans (Amable et al., 2014).

The administration of MSCs-CM in experimental models of diseases has proven to be as effective as the administration of the same MSCs, indicating an essential participation of paracrine mechanisms. Therefore, since the secretome contains the molecules responsible for



**Fig. 6.** Proteomic profile of exosomes derived from CM. (A) Venn diagram showing the number of proteins detected in exosomes from cBM-MSCs (dark grey), cAd-MSCs (light grey) conditioned medium or in common (white numbers). (B) Comparison of biological processes in characterized exosomes proteins identified only in cBM-MSCs (dark grey) and in common (black stripes) of both tissue sources determined by Gene Ontology parameters (n = 3). One protein can be related with different biological functions. Total number of exosomes proteins is the sum of each MSCs source and their common proteins.



**Fig. 7.** Immunomodulatory potential. Number of stained PBMCs after treatment with both MSCs sources (cBM-MSCs in dark grey and cAd-MSCs in light grey), their CMs and exosomes. A decrease in the number of stained PBMCs was observed after direct treatment with cells from both tissue sources and their conditioned media, but this was not observed in the case of exosomes. Data presented as mean and standard error (n = 3). P < 0.05 (\*), P < 0.01 (\*\*), and P < 0.001 (\*\*\*)

the therapeutic action of the MSCs in appreciable quantities, and given its ease of production, scaling and storage, its therapeutic use could have advantages over the cells themselves. This would also eliminate aspects of safety potentially associated with the transplantation of living and proliferating cells, such as immune compatibility, tumorigenicity and the transmission of infections (Makridakis et al., 2013; Villatoro et al., 2017).

On the other hand, intensive production of secretome would be possible through controlled laboratory conditions, providing a convenient source of bioactive factors, which could be evaluated in a similar way to conventional drugs. In the future, it could be modified in its composition for specific cellular effects, through different strategies already consolidated such as cultivation in hypoxia, molecular priming, tridimensional culture, etc. (Vizoso et al., 2017; Ranganath et al., 2012).

From our data and considering differences (better cAd-MSCs proliferative capacity or higher cBM-MSCs secretory profile of some factors), the secretome, for its regenerative, pro-angiogenic and immunomodulatory potential could benefit certain canine pathologies. Since the dog is an interesting preclinical model that naturally suffers from certain pathologies similar to those of humans, the translation of their results with these therapies should be very useful in human medicine (Hoffman and Dow, 2016).

However, new studies to select the most appropriate MSCs-CM for its components according to the pathology to be treated, doses, administration route, as well as its safety and clinical efficacy, would be necessary for its use as a therapeutic element in canine species. Although our study has a limitation due to the small sample size of animal donors and the restriction by the lack of available reagents for the canine species, we believe that our work is a first step in this direction.

### 5. Conclusion

Despite their similarity in certain features and immunomodulatory potential, both MSCs demonstrate, under standard culture conditions, a similar secretion profile, highlighting that cBM-MSCs presented a



higher production capacity in some evaluated factors and the exosomes content.

Therefore, secretome of both type of cells could be good candidate for a possible clinical use in the canine species, considering the clinical application before choosing the source of MSC, since their characteristics condition it.

#### Conflict of interest statement

None.

#### Acknowledgments

This work was partially supported by the Spanish Network on Cell Therapy (Red TerCel, RD16/0011/0022), BIO2015-66266-R (MINECO) and P11-CVI-7245 (Junta de Andalucía). CIBER-BBN is an initiative funded by the VI National R&D&I Plan 2008–2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetimm.2018.12.003>.

#### References

- Al Delfi, I.R., Sheard, J.J., Wood, C.R., Vernallis, A., Innes, J.F., Myint, P., Johnson, W.E.B., 2016. Canine mesenchymal stem cells are neurotrophic and angiogenic: an in vitro assessment of their paracrine activity. *Vet. J.* 217, 10–17.
- Amable, P.R., Teixeira, M.V., Carias, R.B., Granjeiro, J.M., Borojevic, R., 2014. Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly. *Stem Cell Res. Ther.* 5, 53.
- Bearden, R.N., Huggins, S.S., Cummings, K.J., Smith, R., Gregory, C.A., Saunders, W.B., 2017. In-vitro characterization of canine multipotent stromal cells isolated from synovium, bone marrow, and adipose tissue: a donor-matched comparative study. *Stem Cell Res. Ther.* 8, 218.
- Blazquez, R., Sanchez-Margallo, F.M., de la Rosa, O., Dalemans, W., Alvarez, V., Tarazona, R., Casado, J.G., 2014. Immunomodulatory potential of human adipose mesenchymal stem cells derived exosomes on in vitro stimulated T cells. *Front. Immunol.* 5, 556.
- Boomsma, R.A., Geenen, D.L., 2012. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. *PLoS One* 7, 1–8.
- Calle, A., Barrajón-Masa, C., Gómez-Fidalgo, E., Martín-Lluch, M., Cruz-Vigo, P., Sánchez-Sánchez, R., Ramírez, M., 2018. Iberian pig mesenchymal stem/stromal cells from dermal skin, abdominal and subcutaneous adipose tissues, and peripheral blood: in vitro characterization and migratory properties in inflammation. *Stem Cell Res. Ther.* 9, 1–13.
- Carrade, D.D., Borjesson, D.L., 2013. Immunomodulation by mesenchymal stem cells in veterinary species. *Comp. Med.* 63, 207–217.
- Carrade, D.D., Lame, M.W., Kent, M.S., Clark, K.C., Walker, N.J., Borjesson, D.L., 2012. Comparative analysis of the immunomodulatory properties of equine adult-derived mesenchymal stem cells. *Cell Med.* 4, 1–11.
- Chae, H.K., Song, W.J., Ahn, J.O., Li, Q., Lee, B.Y., Kweon, K., Park, S.C., Youn, H.Y., 2017. Immunomodulatory effects of soluble factors secreted by feline adipose tissue-derived mesenchymal stem cells. *Vet. Immunol. Immunopathol.* 191, 22–29.
- Chow, L., Johnson, V., Coy, J., Regan, D., Dow, S., 2017. Mechanisms of immune suppression utilized by canine adipose and bone marrow-derived mesenchymal stem cells. *Stem Cells Dev.* 26, 374–389.
- Colombo, M., Raposo, G., Théry, C., 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* 30, 255–289.
- de Bakker, E., Van Ryssen, B., De Schauwer, C., Meyer, E., 2013. Canine mesenchymal stem cells: state of the art, perspectives as therapy for dogs and as a model for man. *Vet. Q.* 33, 225–233.
- Faltus, T., Brehm, W., 2016. Cell-based veterinary pharmaceuticals - basic legal parameters set by the veterinary pharmaceutical law and the genetic engineering law of the European Union. *Front. Vet. Sci.* 3, 101.
- Fortier, L.A., Travis, A.J., 2011. Stem cells in veterinary medicine. *Stem Cell Res. Ther.* 2, 9.
- Han, C., Sun, X., Liu, L., Jiang, H., Shen, Y., Xu, X., Li, J., Zhang, G., Huang, J., Lin, Z., Xiong, N., Wang, T., 2016. Exosomes and their therapeutic potentials of stem cells. 2015. *Stem Cells Int.* 1–11.
- Haraszti, R., Didiot, M., Sapp, E., Leszyk, J., Shaffer, S., Rockwell, H., Gao, F., Narain, N.R., DiFiglia, M., Kiebish, M.A., Aronin, N., Khvorov, A., 2016. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J. Extracell. Vesicles* 5, 1–14.
- Harman, R., Carlson, K., Gaynor, J., Gustafson, S., Dhupa, S., Clement, K., Hoelzler, M., McCarthy, T., Schwartz, P., Adams, C., 2016. A prospective, randomized, masked, and placebo-controlled efficacy study of intraarticular allogeneic adipose stem cells for the treatment of osteoarthritis in dogs. *Front. Vet. Sci.* 3.
- Harman, R.M., Yang, S., He, M.K., Van de Walle, G.R., 2017. Antimicrobial peptides secreted by equine mesenchymal stromal cells inhibit the growth of bacteria commonly found in skin wounds. *Stem Cell Res. Ther.* 8, 157.
- Hoffman, A.M., Dow, S.W., 2016. Concise review: stem cell trials using companion animal disease models. *Stem Cells* 34, 1709–1729.
- Kang, J., Kang, K., Koo, H., Park, J., Choi, E., Park, Y., 2008. Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. *Stem Cells Dev.* 17, 681–694.
- Katsuda, T., Kosaka, N., Takeshita, F., Ochiya, T., 2013. The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Proteomics* 13, 1637–1653.
- Kim, H.S., Kim, K.H., Kim, S.H., Kim, Y.S., Koo, K.T., Kim, T.I., Seol, Y.J., Ku, Y., Rhyu, I.C., Chung, C.P., Lee, Y.M., 2010. Immunomodulatory effect of canine periodontal ligament stem cells on allogenic and xenogenic peripheral blood mononuclear cells. *J. Periodontol. Implant Sci.* 40, 265–270.
- Kisiel, A.H., McDuffee, L.A., Masaoud, E., Bailey, T.R., Gonzalez, B.P.E., Nino-Fong, R., 2012. Isolation, characterization, and in vitro proliferation of canine mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum. *Am. J. Vet. Res.* 73, 1305–1317.
- Konala, V.B., Mamidi, M.K., Bhonde, R., Das, A.K., Pochampally, R., Pal, R., 2016. The current landscape of the mesenchymal stromal cell secretome: a new paradigm for cell-free regeneration. *Cytotherapy* 18, 13–24.
- Kornicka, K., Śmieczek, A., Węgrzyn, A., Röcken, M., Marycz, K., 2018. Immunomodulatory properties of adipose-derived stem cells treated with 5-Azacytidine and resveratrol on peripheral blood mononuclear cells and macrophages in metabolic syndrome animals. *J. Clin. Med.* 7, 383.
- Kyurkchiev, D., Bochev, I., Ivanova-Todorova, E., Mourdjeva, M., Oreshkova, T., Belemzova, K., Kyurkchiev, S., 2014. Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World J. Stem Cells* 6, 552–570.
- Lee, W.S., Suzuki, Y., Graves, S.S., Iwata, M., Venkataraman, G.M., Mielcarek, M., Peterson, L.J., Ikehara, S., Torok-Storb, B., Storb, R., 2010. Canine bone marrow-derived mesenchymal stromal cells suppress alloreactive lymphocyte proliferation in vitro but fail to enhance engraftment in canine bone marrow transplantation. *Biol. Blood Marrow Transplant.* 17, 465–475.
- Lee, Y., el Andaloussi, S., Wood, M.J.A., 2012. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum. Mol. Genet.* 21, 125–134.
- Lener, T., Gimona, M., Aigner, L., Börger, V., Buzas, E., Camussi, G., Chaput, N., Chatterjee, D., Court, F.A., Del Portillo, H.A., O'Driscoll, L., Fais, S., Falcon-Perez, J.M., Felderhoff-Mueser, U., Fraile, L., Gho, Y.S., Görgens, A., Gupta, R.C., Hendrix, A., Hermann, D.M., Hill, A.F., Hochberg, F., Horn, P.A., de Kleijn, D., Kordelas, L., Kramer, B.W., Krämer-Albers, E.M., Laner-Plamberger, S., Laitinen, S., Leonardi, T., Lorenowicz, M.J., Lim, S.K., Lötvall, J., Maguire, C.A., Marcilla, A., Nazarenko, I., Ochiya, T., Patel, T., Pedersen, S., Pocsfalvi, G., Pluchino, S., Quesenberry, P., Reischl, I.G., Rivera, F.J., Sanzenbacher, R., Schallmoser, K., Slaper-Cortenbach, I., Strunk, D., Tonn, T., Vader, P., van Balkom, B.W., Wauben, M., Andaloussi, S.E., Théry, C., Rohde, E., Giebel, B., 2015. Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. *J. Extracell. Vesicles* 4, 30087.
- Lobb, R.J., Becker, M., Wen Wen, S., Wong, C.S.F., Wiegmanns, A.P., Leimgruber, A., Möller, A., 2015. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J. Extracell. Vesicles* 4, 1–11.
- Maguire, G., 2013. Stem cell therapy without the cells. *Commun. Integr. Biol.* 6, 6.
- Makridakis, M., Roubelakis, M.G., Vlahou, A., 2013. Stem cells: insights into the secretome. *Biochim. Biophys. Acta* 1834, 2380–2384.
- Mathivanan, S., Fahner, C.J., Reid, G.E., Simpson, R.J., 2012. ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res.* 40, 1241–1244.
- Munagala, R., Aqil, F., Jayabalan, J., Gupta, R.C., 2016. Bovine milk-derived exosomes for drug delivery. *Cancer Lett.* 371, 48–61.
- Pawitan, J.A., 2014. Prospect of stem cell conditioned medium in regenerative medicine. *Biomed Res. Int.* 2014.
- Perez-Merino, E.M., Uson-Casaus, J.M., Zaragoza-Bayle, C., Duque-Carrasco, J., Marinasc-Pardo, L., Hermida-Prieto, M., Barrera-Chacon, R., Gualtieri, M., 2015. Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: clinical and laboratory outcomes. *Vet. J.* 206, 385–390.
- Ranganath, S.H., Levy, O., Inamdar, M.S., Karp, J.M., 2012. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 10, 244–258.
- Reiner, A.T., Witwer, K.W., van Balkom, B.W.M., de Beer, J., Brodie, C., Corteling, R.L., Gabriellsson, S., Gimona, M., Ibrahim, A.G., de Kleijn, D., et al., 2017. Concise review: developing best-practice models for the therapeutic use of extracellular vesicles. *Stem Cells Transl. Med.* 6, 1730–1739.
- Russell, K.A., Chow, N.H., Dukoff, D., Gibson, T.W., LaMarre, J., Betts, D.H., Koch, T.G., 2016. Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells. *PLoS One* 11, 1–18.
- Schey, K.L., Luther, J.M., Rose, K.L., 2015. Proteomics characterization of exosome cargo. *Methods* 87, 75–82.
- Schinkothe, T., Bloch, W., Schmidt, A., 2008. In vitro secreting profile of human mesenchymal stem cells. *Stem Cells Dev.* 17, 199–206.
- Screven, R., Kenyon, E., Myers, M.J., Yancy, H.F., Skasko, M., Boxer, L., Bigley, E.C., Borjesson, D.L., Zhu, M., 2014. Immunophenotype and gene expression profile of

- mesenchymal stem cells derived from canine adipose tissue and bone marrow. *Vet. Immunol. Immunopathol.* 161, 21–31.
- Svetlana, D.P., Stratakis, C.A., 2002. Chromosomes: methods for preparation. *Encyclopedia of Life Sciences (ELS)*, first edn. Macmillan Publishers Ltd, Nature Publishing Group, USA, pp. 1–3.
- Takemitsu, H., Zhao, D., Yamamoto, I., Harada, Y., Michishita, M., Arai, T., 2012. Comparison of bone marrow and adipose tissue-derived canine mesenchymal stem cells. *BMC Vet. Res.* 8, 150.
- Tan, L., Wu, H., Liu, Y., Zhao, M., Li, D., Lu, Q., 2016. Recent advances of exosomes in immune modulation and autoimmune diseases. *Autoimmunity* 49, 357–365.
- Toh, W., Zhang, B., Lai, R., LIM, S., 2018. Immune regulatory targets of mesenchymal stromal cell exosomes/small extracellular vesicles in tissue regeneration. *Cytherapy* 2018, 1–8.
- Uranio, M.F., Valentini, L., Lange-Consiglio, A., Caira, M., Guaricci, A.C., L'Abbate, A., Catacchio, C.R., Ventura, M., Cremonesi, F., Dell'Aquila, M.E., 2011. Isolation, proliferation, cytogenetic, and molecular characterization and in vitro differentiation potency of canine stem cells from foetal adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. *Mol. Reprod. Dev.* 78, 361–373.
- Villatoro, A.J., Fernandez, V., Claros, S., Rico-Llanos, G.A., Becerra, J., Andrades, J.A., 2015. Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. *Biomed Res. Int.* 2015.
- Villatoro, A.J., Fernandez, V., Claros, S., Alcoholado, C., Cifuentes, M., Merayo-Lloves, J., Andrades, J.A., Becerra, J., 2017. Regenerative therapies in dry eye disease: from growth factors to cell therapy. *Int. J. Mol. Sci.* 18, 2264.
- Villatoro, A.J., Hermida-Prieto, M., Fernandez, V., Fariñas, F., Alcoholado, C., Rodriguez-García, M., Mariñas-Pardo, L., Becerra, J., 2018a. Allogeneic adipose-derived mesenchymal stem cell therapy in dogs with refractory atopic dermatitis: clinical efficacy and safety. *Vet. Record.*
- Villatoro, A.J., Claros, S., Fernandez, V., Alcoholado, C., Farinas, F., Moreno, A., Becerra, J., Andrades, J.A., 2018b. Safety and efficacy of the mesenchymal stem cell in feline eosinophilic keratitis treatment. *BMC Vet. Res.* 14, 116.
- Vizoso, F.J., Eiro, N., Cid, S., Schneider, J., Perez-Fernandez, R., 2017. Mesenchymal stem cell secretome: toward cell-free therapeutic strategies in regenerative medicine. *Int. J. Mol. Sci.* 18, 1852.