Immunophenotypical characterization of canine mesenchymal stem cells from perivisceral and subcutaneous adipose tissue by a species-specific panel of antibodies

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Abstract: Immunophenotypical characterization of mesenchymal stem cells is fundamental for the design and execution of sound experimental and clinical studies. The scarce availability of species-specific antibodies for canine antigens has hampered the immunophenotypical characterization of canine mesenchymal stem cells (MSC). The aim of this study was to select a panel of species-specific direct antibodies readily useful for canine mesenchymal stem cells characterization.They were isolated from perivisceral and subcutaneous adipose tissue samples collected during regular surgeries from 8 dogs. Single color flow cytometric analysis of mesenchymal stem cells (P3) deriving from subcutaneous and perivisceral adipose tissue, with a panel of 7 direct anti-canine antibodies revealed two largely homogenous cell populations with a similar pattern: CD29+, CD44+, CD73+, CD90+, CD34-, CD45- and MHC-II- with no statistically significant differences among them. Antibody reactivity was demonstrated on canine peripheral blood mononuclear cells. The similarities are reinforced by their in vitro cell morphology, trilineage differentiation ability and RT-PCR analysis (CD90+, CD73+, CD105+, CD44+, CD13+, CD29+, Oct-4+ gene and CD31- and CD45- expression). Our results report for the first time a comparison between the immunophenotypic profile of canine MSC deriving from perivisceral and subcutaneous adipose tissue. The substantial equivalence between the two populations has practical implication on clinical applications, giving the opportunity to choose the source depending on the patient needs. The results contribute to routine characterization of MSC populations grown in vitro, a mandatory process for the definition of solid and reproducible laboratory and therapeutic procedures.

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Abstract

Immunophenotypical characterization of mesenchymal stem cells is fundamental for the design and execution of sound experimental and clinical studies. The scarce availability of species-specific antibodies for canine antigens has hampered the immunophenotypical characterization of canine mesenchymal stem cells (MSC). The aim of this study was to select a panel of species-specific direct antibodies readily useful for canine mesenchymal stem cells ~~MSC~~ characterization. ~~MSC~~ They were isolated from perivisceral ~~(pAT-MSC)~~ and subcutaneous ~~(sAT-MSC)~~ adipose tissue samples collected during regular surgeries from 8 dogs.. Single color flow cytometric analysis of mesenchymal stem cells (P3) deriving from subcutaneous and perivisceral adipose tissue ~~sAT-MSC and pAT-MSC (P3)~~ with a panel of 7 direct anti-canine antibodies revealed two largely homogenous cell populations with a similar pattern: $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD34^-$, $CD45^-$ and MHC-II^- with no statistically significant differences among them. Antibody ~~Antibodies~~ reactivity was demonstrated on canine peripheral blood mononuclear cells. The similarities are reinforced by their *in vitro* cell morphology, trilineage differentiation ability and RT-PCR analysis ($CD90^+$, $CD73^+$, $CD105^+$, $CD44^+$, $CD13^+$, $CD29^+$, Oct-4^+ gene and $CD31^-$ and $CD45^-$ expression). Our results report for the first time a comparison between the

immunophenotypic profile of canine MSC deriving from perivisceral and subcutaneous adipose tissue ~~sAT-MSC and pAT-MSC~~. The substantial equivalence between the two populations has practical implication on clinical applications, giving the opportunity to choose the source depending on the patient needs. The results contribute to routine characterization of MSC populations grown *in vitro*, a mandatory process for the definition of solid and reproducible laboratory and therapeutic procedures.

Key words: mesenchymal stem cells, dog, adipose tissue, species-specific antibody, immunophenotyping

1. Introduction

Regenerative medicine and tissue engineering are promising novel therapeutic approaches in veterinary medicine. In particular, mesenchymal stem cells (MSC) are ~~the most~~ commonly used as a ~~option as a~~ therapeutic tool within the field of cell based therapies and have been the subject of a number of preclinical and clinical studies. Mesenchymal stem cells ~~MSC~~ are self-renewing, multipotent adult stem cells found near blood vessels in different organs such as bone marrow, adipose tissue, blood, umbilical cord, muscle, bone and cartilage. Due to their ability to differentiate into other cell types and to produce a wide range of immunomodulatory, trophic, angiogenic and anti-apoptotic bioactive molecules, MSC have a practical application in the field of cell therapy (Marx et al., 2015). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy defined them as a population of cells that satisfies the following criteria in vitro: 1) adherence to a plastic surface and typical spindle-like shape; 2) ability to differentiate in osteogenic, adipogenic and chondrogenic lineage and 3) the expression of a set of cell surface markers (Dominici et al., 2006).

~~Cell surface marker characterization of human MSC by flow cytometry has recently been carried out (Nery et al., 2013).~~

The expression of cell surface markers on MSC deriving from veterinary species is subject of intense research at the moment. ~~On the contrary, there is little data regarding the expression of cell surface markers on MSC derived from animal species~~.

~~Detailed immunophenotyping analysis of equine MSC from multiple sources was reported, including the expression of CD29, CD44, CD73, CD90, CD105, CD14, CD34, CD45, CD79a, and MHC-II. Although a wide range of markers was included in the study, the interpretation of the results was strongly influenced by the use of anti-human antibodies (Paebst, et al., 2014). Several research groups have published studies about the marker expression of canine MSC isolated from subcutaneous or perivisceral adipose tissue, but the current data are still incomplete. MSC from perivisceral adipose tissue have been characterized as CD90, CD44, CD140a and CD117 positive and CD34 and CD45 negative, however there were no data about the origin of the antibodies that were used (Martinello et al., 2011).~~

~~Another flow cytometric analysis of canine MSC derived from subcutaneous adipose tissue demonstrated a high expression of CD29 and CD44, while CD90 was modestly expressed and there was no expression of CD73, CD34 and CD45. In this case, not all the antibodies used cross-reacted with canine cells and furthermore no control group was included for the evaluation of antibody reactivity (Takemitsu et al., 2012).~~

~~The immunophenotyping profile and gene expression of cell surface markers was further studied on adipose derived MSC that resulted positive for CD44, CD90 and MHC-II; negative for CD29, CD34 and MHC-II using a panel of non-specific canine antibodies (Screven et al., 2014)~~ .

Recently, the dog gained interest as a preclinical/clinical model for cell-based therapies, and canine MSC have been isolated and studied *in vitro*. Furthermore, there is growing interest in the use of MSC in the treatment of injuries and diseases in dogs. Different sources and procedures for the preparation of canine MSC have been proposed and it is difficult to make a clear comparison between the various therapeutic approaches proposed and the clinical outcomes.

Therefore, in order to design and perform robust experimental and clinical studies in veterinary medicine, a fundamental step would be a sound characterization and description of the MSC.

The aim of our study was twofold, (1) to design a panel of canine species specific antibodies able to recognize the markers defining MSC (Dominici et al., 2006), readily useful for the immunophenotypic characterization of canine MSC. (2) To evaluate and compare the immunophenotypic profile of canine adipose-tissue derived MSC (AT-MSC) isolated from subcutaneous and perivisceral fat tissue, using the above defined panel of antibodies. Both information would be useful

for researchers involved in the set-up of cells-based regenerative therapies at both preclinical and experimental level in the dog.

2. Materials and methods

2.1. Study design

Eight client owned canine patients from different breeds, 5 females and 3 males, weighing from 4 to 60 kg, aged between 1,5 and 12 years were selected for adipose tissue sampling during scheduled surgical procedures (Table 1). Patients underwent routine clinical, biochemical and haematological examination prior the surgical procedure. All animal procedures and protocols were performed by licensed veterinary surgeons under standard ethical and sterile conditions. The owners signed an informed consent and agreed on the participation of their dogs.

2.2.Sample collection

Perivisceral and subcutaneous adipose tissue samples were collected during regular surgical procedures from 8 dogs with stable systemic conditions.

After collection each sample was transferred to a vial containing 20 ml of Dulbecco's Eagle Modified Medium (DMEM low glucose, Gibco) additioned with penicillin

(0.10U/ml), streptomycin (0,10 µg/ml) and amphotericin B (2,5 µg/ml, Gibco) until cell isolation (not more than 4 hours). Peripheral blood samples for the isolation and flow cytometry analysis of peripheral blood mononuclear cells were collected from 3 healthy dogs prior to orthopaedic surgery. All the samples were collected following owner consent, under standard ethical and sterile conditions.

2.3.Cell culture

MSC from subcutaneous adipose tissue (sAT-MSC) and perivisceral adipose tissue (pAT-MSC) were isolated from adipose tissue samples weighing 2 grams. The samples were cut with scalpels in small pieces of 0,5 cm diameter and afterwards were transferred in a 15mL Conical Centrifuge *Falcon* Tube containing 10 ml 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; collagenase type I 0,1% p/v). Enzymatic digestion was performed under a mechanical stirrer at 37°C for 45min.

The digested sample was then centrifuged (210xg for 8 min). After removal of the collagenase solution and the fat supernatant, the cell pellet was resuspended in 500µl maintenance medium (DMEM low glucose, Gibco added with 10% fetal bovine serum (FBS), Gibco; penicillin 0,10U/ml, Gibco; streptomycin 0,10µg, Gibco; amfotericine B 2,5 µg/ml,

Gibco) and seeded in 25 cm^2 (Orange Scientific Tissue Culture Flasks) containing 5 ml of DMEM. The cells were maintained in an incubator at 37°C at 5% CO_2. The medium was changed each 2-3 days. Cells were cultured to 80% confluence and then trypsinized with 0,05% Trypsin-EDTA solution (Gibco) The cell culture was grown until passage 3 (P3) when cells were used for the evaluation of the differentiation capacity, flow cytometric analysis and RT-PCR.

2.4.Tri-lineage cell differentiation

1. Adipogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at P3 were seeded in six-well plates at a density of 6 x 10^3 cells/cm^2. In each well, 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO_2. At a confluency of 80% they were treated with adipogenic differentiation media (StemPro Adipogenesis Differentiation Kit). The flasks were then put in an incubator with humidified atmosphere of 5% CO_2 and temperature of 37°C. Medium was changed each 2-3 days. After 21 days the cells were fixed with 70% ethanol and stained with Oil Red O coloration.

2. Chondrogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at passage 3 were seeded in six-well plates at a density

of 6 x 10^3 cells/cm^2. In each well 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO_2. At a confluency of 80% they were treated with with chondrogenic differentiation mediun (StemPro Chondrogenesis Differentiation Kit). The flasks were then put in an incubator with humidified atmosphere of 5% CO_2 and temperature of 37 °C. Medium was changed each 2-3 days. After 21 days the cells were fixed with 4% formaldehyde and stained with Alcian blue.

3. Osteogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at passage 3 were seeded in six-well plates at a density of 6 x 10^3 cells/cm2. In each well 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO2. Arriving at confluency of 80%, the cells were treated with osteogenic induction medium (DMEM additioned with 100nM dexamethasone, 10µM glycerophosphate and 0.250 mM ascorbic acid). 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).

2.5.Immunophenotyping

Single color flow cytometry analysis was performed for the assessment of surface marker expression of cells from both sources of adipose tissue. The expression of the following

markers was evaluated: CD29, CD34, CD44, CD45, CD73, CD90 and MHC-II, using a panel of seven antibodies (Table 1).

At P3, MSC were trypsinized (0,05% Trypsin-EDTA, Gibco) and centrifuged at 210xg for 8 minutes. They were then resuspended in 3 ml of complete medium for cell count using a Burker's hemocytometer. Then, 2.5 x 10^4 cells were transferred in conical and round bottom tubes for flow cytometry analysis, washed with 1 ml sterile PBS supplemented with 1% fetal bovine serume (FBS), and then centrifuged at 210x g for 8 minutes. The supernatant was eliminated and, in every tube, 5 µl of each antibody was added.

For every sample, one tube containing the same number of unmarked cells was evaluated as a negative control.

After dark incubation for 15 minutes at room temperature, 1ml of PBS was added and the cells were centrifuged at 210xg for 8 minutes. The pellet was then added 0,5ml of PBS and evaluated by flow cytometry analysis (Cytomics FC 500, Beckman Coulter). Dead cells were excluded using SytoxAAdvanced Dead Cell Stain Kit (Life Technologies), according to the manufacturer's instructions.

Antibodies were tested on peripheral blood mononuclear cells (PBMC) of 3 healthy dogs to confirm reactivity. PBMC were isolated from 2 ml of 3 dog blood samples collected in lithium–heparin as anticoagulant by density gradient in Histopaque-

1077 solution (Sigma, St. Louis, MO) according to the manufacturer's instructions. Blood samples were taken and then stratified on Histopaque-1077 solution (1:1, v/v, Sigma) and centrifuged at 400 × g for 30 min; purified PBMC were washed with sterile Phosphate Buffer Solution (PBS) (Sigma) supplemented with 1% fetal FBS and resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM l-glutamine, 100mM non-essential amino-acids, 50 mM 2-mercaptoethanol (Sigma) and 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

Cells were counted by inverted optical microscope and concentration was assessed before being used for surface staining flow cytometry analysis. 400,000 cells were mixed with 5µl of the specific antibody for each surface antigen in a plastic tube, after an incubation for 15' in the dark at room temperature, cells were washed with PBS with 1% FBS, centrifuged for 5' at 400xg and re-suspended in 0.5 ml of PBS/1%FCS and finally set aside for flow cytometry analysis (Cytomics FC 500, Beckman Coulter).

2.6.RT-PCR

Assessment of the phenotypic expression of different cell markers was performed by Reverse-Transcriptase-PCR (RT-PCR). Total RNA was extracted from $1.5x10^6$ cells at P3 (80% of confluence) using the kit Nucleospin® RNA II (Macherey-

281 Nagel) following manufacturer's instructions. cDNA was then
282 obtained via reverse transcription of 1.5µg of total RNA using
283 RevertAit™ First Strand cDNA Synthesis Kit (Fermentas).
284 PCR was performed using 2µl of cDNA. The final PCR
285 mixture contained 1x amplification buffer with 2.5 mM $MgCl_{2,}$
286 10mM dNTP Mix (Thermo Scientific), 0,25µm specific
287 forward and reverse primers, 1 U Dream Taq (Thermo
288 Scientific) in a final volume of 25µl.

289 Table 2 reports the list of genes whose expression was
290 analyzed, their gene accession number, the sequence of forward
291 and reverse specific set of primers and the length of the relative
292 amplicons. All PCRs were performed using the following
293 protocol: denaturation at 94°C for 30 sec; annealing at 55°C for
294 30 sec; extension at 72°C for 30 sec, with 35 cycles The
295 products of the RT-PCR were separated on agarose gel (1,5%
296 P/V) in TAE buffer stained with 3,5µl ethidium bromide
297 (10mg/ml). Amplicons were visualized under UV light with a
298 trans-illuminator and images acquired by a Canon digital
299 camera. The analysis was repeated with two different replicates
300 for each tissue sample. A semi-quantitative analysis of the
301 expression of MSCs markers was performed evaluating the
302 optical density of each positive band by means of the ImageJ
303 image processing software normalized to the expression of the
304 housekeeping gene GAPDH.

305 *2.7.Statistical analysis*

The results of the immunophenotypic characterization of MSC 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. All the tests were made using SPSS 16 IBM software.

3. Results

3.1.Cell culture and isolation

After 24 – 48 hours, cells were adherent to the surface of the flasks. Medium was changed after two-three days in order to eliminate non-adherent cells. During their growth, cells appeared as a homogeneous population of typical fibroblast-like cell morphology with elongated spindle like shape.

3.2.Tri-lineage cell differentiation

Cell cultures at P3 ~~have~~ were able to differentiate into osteogenic, adipogenic and chondrogenic lineages. ~~A large number of~~ Cells stimulated with adipogenic medium contained intracellular lipid vacuoles stained with Oil Red O, confirming the adipogenic differentiation of sAT-MSC and pAT-MSC, following the appropriate stimulation in vitro. Cells stimulated with osteogenic induction medium formed aggregates and had ~~numerous~~ extracellular calcium deposits, as by with von Kossa

staining confirmed the osteogenic differentiation of cells from both sources. The cell cultures treated with chondrogenic medium contained aggregates of proteoglycans stained in blue with Alcian Blue coloration. Control groups for each treatment showed no evidence of differentiation (Fig. 1).

3.3.FACS analysis

All antibodies tested on canine PBMC showed the expected reactivity (data not shown). For immunophenotypical analysis, the live population of MSC was gated in the scatter plot for further fluorescence intensity analysis in the histogram plot (Fig. 2).

Our data showed that MSC at P3 deriving from perivisceral and subcutaneous adipose tissue were positive for: CD29, CD44, CD73, CD90; and negative for CD34, CD45 and MHC-II (Tables 3, 4). There was no significant difference in surface antigen expression between the two groups of MSC.

3.4.Reverse transcription analysis of gene expression

Expression analysis of a panel of genes consisting of CD90, CD73, CD105, CD45, CD34, CD44, CD13, CD29, CD31 and Oct-4 was performed on sAT-MSC and pAT-MSC at P3. Both populations were negative for CD45 and CD31. pAT-MSC were also negative for CD34 expression, while sAT-MSC showed weak expression. All other genes of the panel were

expressed in both sAT-MSC and pAT-MSC. Semiquantitative analysis of marker profile, demonstrated a substantial equivalence between the two cell populations (Table 5).

4. Discussion

MSC-based treatment has been increasingly applied in veterinary medicine in the recent years. Several studies have reported the beneficial effects for different pathological states in dogs (Black et al., 2008, Hall et al., 2010, Pogue et al., 2013, Alamoudi et al., 2014, Marx et al., 2014, Penha et al., 2014, Villatoro et al., 2015, Perez-Merino et al., 2015, Kim et al., 2015 and Lee et al., 2015). ~~horses (Del Bue et al., 2008, Rich et al, 2014 and Govoni et al, 2015) and cats (Trzil, et al., 2015, Quimby, et al., 2016).~~ 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 MSC and for the safety of clinical treatments based on these cells, it is necessary to have accurate cell characterization. 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.

Following the basic criteria for the characterization of human MSC (Dominici et al., 2006), equivalent MSC derived from laboratory and domestic animals were demonstrated to have fibroblast-like shape, ability to differentiate into osteogenic, chondrogenic and adipogenic lineages and to have a determinate cell surface antigen pattern. ~~However, the lack of species-specific antibodies has been a major drawback towards the full and accurate immunophenotypic characterization of animal derived MSC.~~ ~~Therefore,~~ The first aim of our study was to select a panel of species-specific antibodies for the evaluation of cell surface CD marker ~~markers~~ expression in canine adipose tissue derived MSC 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 MSC, since they have

become an attractive cell therapy product for small as well as large animal veterinary practitioners.

A further aim was to compare the marker expression of MSC derived from subcutaneous and perivisceral adipose tissue. Indeed, the characterization of these two populations of MSC would be of practical relevance for their clinical use. Subcutaneous adipose tissue is an easily available source of MSC. Samples of a few grams of fat tissue 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 MSC for their applications.

MSC derived from the two tissue sources were analysed 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). ~~Cells~~ MSC 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

423 osteogenic, chondrogenic and adipogenic lineages when
424 stimulated with appropriate induction medium. A previous
425 study reported similar results (Guercio et al., 2013), but lacked
426 the immunophenotypic characterization of the cell population.
427 Thus, in the present study, we chose to determine the
428 expression of a panel of cell surface CD markers (CD29, CD34,
429 CD44, CD45, CD90, CD73 and MHC-II), typical for the MSC
430 (Dominici et al., 2006) using species-specific anti-canine
431 antibodies.

432 CD 105 - one of the three surface markers that define human
433 MSC (Dominici et al., 2006), was not taken in consideration in
434 our study since we were not able to find any commercially
435 available anti canine - CD105 antigen. ~~canine antibodies.~~
436 However, by means of RT-PCR we analysed the CD105
437 expression and it was found positive in both sAT-MSC and in
438 pAT-MSC.

439 Antibody reactivity was confirmed by flow cytometry of canine
440 PBMC (Peripheral Blood Mononucleated Cells) that served as
441 a positive control. Interestingly, canine AT-MSC derived from
442 subcutaneous and perivisceral adipose tissue showed a similar
443 immunophenotypic pattern, which from a practical point of
444 view indicates that both tissues can be used as a valid source
445 for the isolation of MSC. Both cell populations can be defined
446 as $CD29^{+}CD44^{+}CD90^{+}CD45^{-}CD34^{-}CD73^{-}MHC\text{-}II^{-}$. As far as
447 we know, this is the first study that characterized cAT-MSC by

using a panel of canine species specific antibodies for both positive as well as negative markers. We are aware that culture conditions influence the immunophenotypic profile of MSC, as it has been demonstrated for example, by bFGF (Gharibi and Hughes, 2012). Therefore for each future characterization in different culture conditions it is recommended to take this fact in consideration. Different researchers included anti-human antibodies in their protocols, however, they cannot be considered fully reliable for the characterization of canine MSC. Rozemuller et al., 2010 studied the expression of surface cell surface marker expression on canine BM-MSC using a panel of 43 anti-human antibodies. 24 of them, among which CD73, CD90 and CD105 did react with human MSC, but failed to cross-react with canine MSC, therefore cannot be considered adequate for the definition of the immune phenotype of canine MSC.. Takemitsu et al., isolated and characterized canine AT-MSC 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 conseguence to the use of a nonspecific canine antibody.

By RT-PCR analysis, we analyzed 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-MSC as well as pAT-MSC, as it has already been described in canine (Neupane et al., 2008, Reich et al., 2012) and human (Han et al., 2014) AT-MSC. Furthermore, the cells resulted positive for CD13 and negative for CD 31. Although we did not evaluate CD105 expression by flow cytometry analysis, the RT-PCR revealed a positive expression for both sAT-MSC and pAT-MSC. As a whole 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. According to Takemitsu et al. (2012), and Screven et al. (2014), canine AT-MSC do not express CD34. However Russell et al. (2016) found a moderately high expression of CD34 on AT-MSC, explaining that actually the fat harvesting site does have an influence on surface markers expression. We indeed detected a positive, although low expression of CD34 at mRNA level in sAT-MSC. On the other hand at a protein level CD34 was absent. This finding could be explained with the observation that sometimes low level of gene transcription does not result in a significant protein synthesis (Screven et al., 2014).

The comparison of the immunophenotypical profile of AT-MSC among canine, human and other species adipose tissue

498 derived MSC would be of interest, as the dog is considered a
499 suitable animal model for the study of human diseases
500 (Hoffman and Dow, 2016).

501 In comparison to laboratory animals, canine anatomy and
502 physiology, as well as the prevalence in this species of natural
503 occurring diseases with similar pathogenesis, more closely
504 resemble humans. Dogs have a longer life-span, live in
505 environments similar to humans and therefore are exposed to
506 different external factors that are part of the aetiology of
507 common diseases and conditions such as diabetes, obesity and
508 tumors. Furthermore, the clinical follow-up is similar and there
509 is a wide range of imaging techniques developed for veterinary
510 patients (Volk and Theoret, 2013) suitable to provide solid
511 knowledge about the clinical evolution of the diseases.

512 A complete overlap of marker expression between human MSC
513 has yet to be demonstrated due to the lack of an extensive cell
514 characterization in some species. However, similarities can be
515 found between MSCs derived from domestic and laboratory
516 animals and humans. Most of the markers analyzed in this
517 study share a similar distribution in the different species, with
518 the exception of CD73. We encountered a similar ~~identical~~
519 expression of CD29, CD34, CD44, CD45, CD90 and MHC-II
520 which could suggest that *in vitro* cultured canine MSC
521 possibly share a similar immunophenotypic profile with human
522 MSC (Takemitsu et al., 2012; Nery et al., 2012). The unique

523 difference found was in the expression of CD73, one of the
524 three positive markers proposed for defining hMSC (≥95%)
525 (Dominici et al., 2006). Interestingly, we report here for the
526 first time that the expression of CD73 is lower in other animal
527 ~~an animal~~ species compared to humans (Table 6). This would
528 suggest that there is a slightly different immunophenotypic
529 profile that should be taken in consideration for the correct
530 interpretation and characterization of AT-MSC in veterinary
531 patients.

532 Bone marrow has been commonly used in veterinary medicine
533 as a source of MSC. Their immunophenotype has been
534 described in different works. Malagola et al., (2016) by FACS
535 analysis characterized cBM-MSC as
536 $CD105^{+}CD90^{+}CD166^{+}CD29^{+}CD45^{-}$. Additionally, Takemitsu
537 et al. (2012) and Screven et al., (2014) compared the
538 immunophenotypic profile of cAT-MSC and cBM-MSC, which
539 resulted to be similar. However Screven et al., did not report
540 any statistical analysis and compared cells collected at a
541 different in vitro passage. Takemtsu et al., compared cells
542 deriving from dogs having the same gender, similar age and
543 weight, and bred in similar conditions. In the present work, on
544 the other hand we have isolated cAT-MSC from canine patients
545 having different age, gender, weight and living in different
546 environments. Therefore, we compared our results with the

immunophenotypical characteristics previously described for bone marrow derived MSC Takemitsu et al., 2012. (Table 7). Both profiles are similar in terms of CD29 and CD44 as positive markers and CD34, CD45 and MHC-II as negative markers. A difference exists in the expression of CD73 and CD90, which appears to be lower in BM-MSC. However, the authors used noncanine-specific antibodies for the analysis, which can have an influence on the interpretation of the percentage of expression. Altogether, the results presented in Table 7 indicate a quite similar profile between canine adipose tissue and bone marrow derived MSC.

5. Conclusions

In the present paper we suggest a panel of antibodies specific to canine antigens suitable for the characterization of canine MSC by cytofluorimetry, i.e. CD29, CD34, CD44, CD45, CD90, CD73 and MHC-II. Using this panel of antibodies, we demonstrated that MSC derived from subcutaneous and perivisceral fat tissue samples shares an overlapping immune-profile, suggesting that the choice of the source of adipose tissue collected to expand MSC to be used in clinical practice, could be decided by the veterinarian of the basis of clinical considerations and not as a consequence of biological difference between cells derived from the two different sources. Furthermore, the

immunophenotype described by our panel of antibodies also suggests that adipose tissue derived MSC and bone marrow derived MSC show a similar profile. However, we would like to point out that additional studies are necessary to broaden the above listed panel of antibodies, in order to obtain a more accurate characterization of the immunophenotype of MSC.

Conflict of interest statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Canine patients' donors of adipose tissue samples

Patient	Gender	Age	Breed	Weight	Sampling collection location
Dog 1	Female	1.5 years	Mixed-breed	10kg	Tela subserosa of the perimetrium
Dog 2	Female	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

608 **Table 2. Description of the panel of antibodies**

Target 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

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627 **Table 3. Markers used for the evaluation of cell surface**
628 **markers expression of MSC by RT-PCR**

Markers	Accession Number	Primers	Amplicon size
CD 13	NM_001146034.1	Fw: GGTCCTTACCATCACCTGGC Rv: CCTAAGGCCATCCATCGTCC	335bp
CD 29	XM_005616949.1	Fw: AGGATGTTGACGACTGCTGG Rv: ACCTTTGCATTCAGTGTTGTGC	356bp
CD 31	XM_848326	Fw: GCCCGAAGTTCACTCTCAAG Rv:CACTCCTTTGACCCACACCT	410bp
CD 34	NM_001003341.1	Fw: GAGATCACCCTAACGCCTGG Rv: GGCTCCTTCTCACACAGGAC	383bp
CD 44	NM_001197022.1	Fw: CCCATTACCAAAGACCACGA Rv: TTCTCGAGGTTCCGTGTCTC	408bp
CD 45	XM_005622282.1	Fw: TGTTTCCAGTTCTGTTTCCCCA Rv: TCAGGTACAAAGCCTTCCCA	432bp
CD 73	XM_532221.4	Fw: GATGGGAAAGGCAAGAGGCT Rv: TTCCTGGCATCTTGCTACGG	317bp
CD 90	NM_001287129.1	Fw: AAGCCAGGATTGGGGATGTG Rv: TGTGGCAGAGAAAGCTCCTG	285bp
CD 105	XM_005625330.2	Fw: GCTGAGGACAGAGATGA	421bp

			CCA Rv: CACGGAGGAGGAAGCTG AAG	
	Oct-4	XM_538830.1	Fw: AAGCCTGCAGAAAGACCT G Rv: GTTCGCTTTCTCTTTCGGG C	286bp

Table 4. Flow cytometric analysis of adipose tissue derived MSC

Markers	sAT-MSC [a]	pAT-MSC [b]
CD 29	99.5%±0.6	99.2%±1.5
CD 34	0.5%±1	0.25%±0.5
CD 44	78%±17	76.7%±18
CD 45	0.25%±0.5	0.5%±0.6
CD 73	14%±12.3	17%±14
CD 90	89%±6.7	79.5%±7.1
MHC-II	4.5%±6.3	4.5%±6.4

a and b: Data are displayed as percentages expressed as mean ± SD. Mood's median test was applied and no statistical difference for $P<0.05$ was found.

Table 5. Summary of cell surface markers expression measured by flow cytometry analysis

Cells	CD29	CD34	CD44	CD45	CD73	CD90	MHC-II
sAT-MSC	+++ [b]	- [c]	+++ [b]	- [c]	++ [a]	+++ [b]	- [c]
pAT-MSC	+++ [b]	- [c]	+++ [b]	- [c]	++ [a]	+++ [b]	- [c]
PBMC	+++ [b]	+++ [b]	+++ [b]	+++ [b]	+++ [b]	+++ [b]	+++ [b]

a ++ (10-40% positive cells)

b +++ (>40% positive cells)

c - (<5% positive cells).

Different groups are classified as described by Screven et al 2014

Table 6. Gene expression analysis of pAT-MSC and sAT-MSC using semi-quantitative RT-PCR

Cell marker	pAT-MSC	sAT-MSC
CD90	+++ [d]	+++ [d]
CD73	++ [c]	++ [c]
CD105	++ [c]	++ [c]
CD45	- [a]	- [a]
CD34	- [a]	+ [b]
CD44	+++ [d]	+++ [d]
CD13	+++ [d]	+++ [d]
CD29	+++ [d]	+++ [d]
CD31	- [a]	- [a]
Oct-4	+ [b]	+ [b]

a – (not expressed)

b + (<25% of GAPDH signal)

c ++ (25-50% of GAPDH signal)

d +++ (>50% of GAPDH signal)

Table 7. Comparison of the immunophenotypic profile of adipose derived MSC from multiple species

Species	Marker expression						
	CD 29	CD 34	CD 44	CD 45	CD 73	CD 90	MHC-II
Human[e]	+++ [c]	- [a]	+++ [c]	- [a]	+++ [c]	+++ [c]	- [a]
Mouse[f]	+++ [c]	/ [d]	+++ [c]	- [a]	++ [b]	+++ [c]	/ [d]
Rat[g]	+++ [c]	- [a]	/ [d]	- [a]	/ [d]	/ [d]	/ [d]
Dog[h]	+++ [c]	- [a]	+++ [c]	- [a]	++ [a]	+++ [c]	- [a]
Horse[i]	/ [d]	- [a]	+++ [c]	- [a]	- [a]	+++ [c]	- [a]
Cat[j]	/ [d]	- [a]	+++ [c]	- [a]	/ [d]	+++ [c]	/ [d]

a - (<5% positive cells)

b ++ (10-40% positive cells)

c +++ (>40% positive cells)

d / (not known)

Different groups are classified as described by Screven et al 2014

e (Dominici et al., 2006)

f (Taha and Hedayati, 2010) (Laschke et al., 2013)

g (Lotfy et al., 2014) (Tapp et al., 2008)

h *our results*

i (de Mattos Carvalho et al., 2009)(Pascucci et al., 2011)(Barberini et al., 2014)

j (Kono et al., 2014)

Table 8. Comparison of the expression of the immunophenotypic profile between MSC deriving from subcutaneous adipose tissue, perivisceral adipose tissue and bone marrow

Markers	sAT-MSC	pAT-MSC	BM-MSC[a]
CD 29	99.5%±0.6	99.2%±1.5	98.41±0.53
CD34	0.5%±1	0.25%±0.5	0.88±0.21
CD 44	78%±17	76.7%±18	98.90±0.25
CD 45	0.25%±0.5	0.5%±0.6	0.24±0.07
CD 73	14%±12.3	17%±14	0.0081±0.01
CD 90	89%±6.7	79.5%±7.1	19.10±2.10
MHC-II	4.5%±6.3	4.5%±6.4	2.85±1.35

[a] Takemitsu et al., 2012

Figure 1 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**) (40X, scale bar 200µm). 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**) (10X, scale bar 100µm). 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**) (10X, scale bar 100um).

Figure 2 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 (a) contains data regarding the MSC deriving from subcutaneous adipose tissue and the low panel (b) data about MSC 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.

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Highlights

- The design of MSC based therapeutic protocols requires an accurate cell characterization
- Selection of a panel of 7 species specific anti-canine antibodies for flow cytometric characterization of canine AT-MSC
- Canine AT-MSC from subcutaneous and perivisceral adipose tissue have a similar immunophenotypic profile
- Comparison of the immunophenotypical profile of AT-MSC from veterinary species, laboratory animals and human origin
- AT-MSC derived from animal species have a lower CD73 expression compared to human AT-MSC

Immunophenotypical characterization of canine mesenchymal stem cells from perivisceral and subcutaneous adipose tissue by a species-specific panel of antibodies

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Abstract

Immunophenotypical characterization of mesenchymal stem cells (MSC) is fundamental for the design and execution of sound experimental and clinical studies. The scarce availability of species-specific antibodies for canine antigens has hampered the immunophenotypical characterization of canine MSC. The aim of this study was to select a panel of species-specific direct antibodies readily useful for canine MSC characterization. MSC isolated from perivisceral (pAT-MSC) and subcutaneous (sAT-MSC) adipose tissue samples collected during regular surgeries from 8 dogs, were cultured in vitro under standard conditions. Single color flow cytometric analysis of sAT-MSC and pAT-MSC (P3) with a panel of 7 direct anti-canine antibodies revealed two largely homogenous cell populations with a similar pattern: $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD34^-$, $CD45^-$ and MHC-II^- with no statistically significant differences among them. Antibodies reactivity was demonstrated on canine peripheral blood mononuclear cells. The similarities are reinforced by their *in vitro* cell morphology, trilineage differentiation ability and RT-PCR analysis ($CD90^+$, $CD73^+$, $CD105^+$, $CD44^+$, $CD13^+$, $CD29^+$, Oct-4^+ gene and $CD31^-$ and $CD45^-$ expression). Our results report for the first time a comparison between the immunophenotypic profile of canine sAT-MSC and pAT-MSC. The substantial equivalence between the two populations has practical implication on clinical

applications, giving the opportunity to choose the source depending on the patient needs. The results contribute to routine characterization of MSC populations grown *in vitro*, a mandatory process for the definition of solid and reproducible laboratory and therapeutic procedures.

Key words: mesenchymal stem cells, dog, adipose tissue, species-specific antibody, immunophenotyping

1. Introduction

Regenerative medicine and tissue engineering are promising novel therapeutic approaches in veterinary medicine. In particular, mesenchymal stem cells (MSC) are the most common option as a therapeutic tool and have been the subject of a number of preclinical and clinical studies. MSC are self-renewing, multipotent adult stem cells found near blood vessels in different organs such as bone marrow, adipose tissue, blood, umbilical cord, muscle, bone and cartilage. Due to their ability to differentiate into other cell types and to produce a wide range of immunomodulatory, trophic, angiogenic and anti-apoptotic bioactive molecules, MSC have a practical application in the field of cell therapy (Marx et al., 2015).

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy defined them as a population of cells that satisfies the following criteria in vitro: 1) adherence to a plastic surface and typical spindle-like shape; 2) ability to differentiate in osteogenic, adipogenic and chondrogenic lineage and 3) the expression of a set of cell surface markers (Dominici et al., 2006).

Cell surface marker characterization of human MSC by flow cytometry has recently been carried out (Nery et al., 2013).

On the contrary, there is little data regarding the expression of cell surface markers on MSC derived from animal species.

Detailed immunophenotyping analysis of equine MSC from multiple sources was reported, including the expression of CD29, CD44, CD73, CD90, CD105, CD14, CD34, CD45, CD79a, and MHC-II. Although a wide range of markers was included in the study, the interpretation of the results was strongly influenced by the use of anti-human antibodies (Paebst, et al., 2014). Several research groups have published studies about the marker expression of canine MSC isolated from subcutaneous or perivisceral adipose tissue, but the current data are still incomplete. MSC from perivisceral adipose tissue have been characterized as CD90, CD44, CD140a and CD117 positive and CD34 and CD45 negative, however there were no data about the origin of the antibodies that were used (Martinello et al., 2011).

Another flow cytometric analysis of canine MSC derived from subcutaneous adipose tissue demonstrated a high expression of CD29 and CD44, while CD90 was modestly expressed and there was no expression of CD73, CD34 and CD45. In this case, not all the antibodies used cross-reacted with canine cells and furthermore no control group was included for the evaluation of antibody reactivity (Takemitsu et al., 2012).

The immunophenotyping profile and gene expression of cell surface markers was further studied on adipose derived MSC that resulted positive for CD44, CD90 and MHC-II; negative

for CD29, CD34 and MHC-II using a panel of non-specific canine antibodies (Screven et al., 2014) .

There is growing interest in the use of MSC in the treatment of injuries and diseases in dogs. Different sources and procedures for the preparation of canine MSC have been proposed and it is difficult to make a clear comparison between the various therapeutic approaches proposed and the clinical outcomes.

Therefore, in order to design and perform robust experimental and clinical studies in veterinary medicine, a fundamental step would be a sound characterization and description of the MSC.

The aim of the present work was to select a panel of species-specific direct antibodies that can be readily used for the characterization of canine MSC. In particular, taking in consideration the relatively easy and low invasive procedures needed to collect tissue samples, we chose to perform the immunophenotype characterization of adipose tissue derived MSC. Using a defined panel of antibodies, we compared MSC deriving from two different adipose tissue sources, i.e. subcutaneous and perivisceral adipose tissue.

2. Materials and methods

2.1.Sample collection

Perivisceral and subcutaneous adipose tissue samples were collected during regular surgical procedures from 8 dogs with stable systemic conditions.

After collection each sample was transferred to a vial containing 20 ml of Dulbecco's Eagle Modified Medium (DMEM low glucose, Gibco) additioned with penicillin (0,10U/ml), streptomycin (0,10 µg/ml) and amphotericin B (2,5 µg/ml, Gibco) until cell isolation (not more than 4 hours). Peripheral blood samples for the isolation and flow cytometry analysis of peripheral blood mononuclear cells were collected from 3 healthy dogs prior to orthopaedic surgery. All the samples were collected following owner consent, under standard ethical and sterile conditions.

2.2.Cell culture

MSC from subcutaneous adipose tissue (sAT-MSC) and perivisceral adipose tissue (pAT-MSC) were isolated from adipose tissue samples weighing 2 grams. The samples were cut with scalpels in small pieces of 0,5 cm diameter and afterwards were transferred in a 15mL Conical Centrifuge *Falcon* Tube containing 10 ml 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; collagenase type I 0,1% p/v). Enzymatic digestion was performed under a mechanical stirrer at 37°C for 45min.

The digested sample was then centrifuged (210xg for 8 min). After removal of the collagenase solution and the fat supernatant, the cell pellet was resuspended in 500µl maintenance medium (DMEM low glucose, Gibco added with 10% fetal bovine serum (FBS), Gibco; penicillin 0,10U/ml, Gibco; streptomycin 0,10µg, Gibco; amfotericine B 2,5 µg/ml, Gibco) and seeded in 25 cm^2 (Orange Scientific Tissue Culture Flasks) containing 5 ml of DMEM. The cells were maintained in an incubator at 37°C at 5% CO_2. The medium was changed each 2-3 days. Cells were cultured to 80% confluence and then trypsinized with 0,05% Trypsin-EDTA solution (Gibco) The cell culture was grown until passage 3 (P3) when cells were used for the evaluation of the differentiation capacity, flow cytometric analysis and RT-PCR.

2.3.Tri-lineage cell differentiation

1. Adipogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at P3 were seeded in six-well plates at a density of 6 x 10^3 cells/cm^2. In each well, 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO_2. At a confluency of 80% they were treated with adipogenic differentiation media

(StemPro Adipogenesis Differentiation Kit). The flasks were then put in an incubator with humidified atmosphere of 5% CO_2 and temperature of 37°C. Medium was changed each 2-3 days. After 21 days the cells were fixed with 70% ethanol and stained with Oil Red O coloration.

2. Chondrogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at passage 3 were seeded in six-well plates at a density of 6 x 10^3 cells/cm^2. In each well 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO_2. At a confluency of 80% they were treated with with chondrogenic differentiation mediun (StemPro Chondrogenesis Differentiation Kit). The flasks were then put in an incubator with humidified atmosphere of 5% CO_2 and temperature of 37 °C. Medium was changed each 2-3 days. After 21 days the cells were fixed with 4% formaldehyde and stained with Alcian blue.

3. Osteogenic differentiation

MSC deriving from subcutaneous and perivisceral adipose tissue, at passage 3 were seeded in six-well plates at a density of 6 x 10^3 cells/cm2. In each well 3 ml of DMEM was added and the cells were incubated at 37°C and 5% CO2. Arriving at confluency of 80%, the cells were treated with osteogenic induction medium (DMEM additioned with 100nM dexamethasone, 10µM glycerophosphate and 0.250 mM

ascorbic acid). 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).

2.4.Immunophenotyping

Single color flow cytometry analysis was performed for the assessment of surface marker expression of cells from both sources of adipose tissue. The expression of the following markers was evaluated: CD29, CD34, CD44, CD45, CD73, CD90 and MHC-II, using a panel of seven antibodies (Table 1).

At P3, MSC were trypsinized (0,05% Trypsin-EDTA, Gibco) and centrifuged at 210xg for 8 minutes. They were then resuspended in 3 ml of complete medium for cell count using a Burker's hemocytometer. Then, 2.5 x 10^4 cells were transferred in conical and round bottom tubes for flow cytometry analysis, washed with 1 ml sterile PBS and then centrifuged at 210x g for 8 minutes. The supernatant was eliminated and, in every tube, 5 µl of each antibody was added.

For every sample, one tube containing the same number of unmarked cells was evaluated as a negative control.

After dark incubation for 15 minutes at room temperature, 1ml of PBS was added and the cells were centrifuged at 210xg for 8 minutes. The pellet was then added 0,5ml of PBS and evaluated by flow cytometry analysis (Cytomics FC 500, Beckman

Coulter). Dead cells were excluded using SytoxAAdvanced Dead Cell Stain Kit (Life Technologies), according to the manufacturer's instructions.

Antibodies were tested on peripheral blood mononuclear cells (PBMC) of 3 healthy dogs to confirm reactivity. PBMC were isolated from 2 ml of 3 dog blood samples collected in lithium–heparin as anticoagulant by density gradient in Histopaque-1077 solution (Sigma, St. Louis, MO) according to the manufacturer's instructions. Blood samples were taken and then stratified on Histopaque-1077 solution (1:1, v/v, Sigma) and centrifuged at 400 × g for 30 min; purified PBMC were washed with sterile Phosphate Buffer Solution (PBS) (Sigma) supplemented with 1% fetal bovine serum (FBS) and resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM l-glutamine, 100mM non-essential amino-acids, 50 mM 2-mercaptoethanol (Sigma) and 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

Cells were counted by inverted optical microscope and concentration was assessed before being used for surface staining flow cytometry analysis. 400,000 cells were mixed with 5µl of the specific antibody for each surface antigen in a plastic tube, after an incubation for 15' in the dark at room temperature, cells were washed with PBS with 1% FBS,

centrifuged for 5’ at 400xg and re-suspended in 0.5 ml of PBS/1%FCS and finally set aside for flow cytometry analysis (Cytomics FC 500, Beckman Coulter).

2.5.RT-PCR

Assessment of the phenotypic expression of different cell markers was performed by Reverse-Transcriptase-PCR (RT-PCR). Total RNA was extracted from $1.5x10^6$ cells at P3 (80% of confluence) using the kit Nucleospin® RNA II (Macherey-Nagel) following manufacturer’s instructions. cDNA was then obtained via reverse transcription of 1.5µg of total RNA using RevertAit™ First Strand cDNA Synthesis Kit (Fermentas). PCR was performed using 2µl of cDNA. The final PCR mixture contained 1x amplification buffer with 2.5 mM $MgCl_2$, 10mM dNTP Mix (Thermo Scientific), 0,25µm specific forward and reverse primers, 1 U Dream Taq (Thermo Scientific) in a final volume of 25µl.

Table 2 reports the list of genes whose expression was analyzed, their gene accession number, the sequence of forward and reverse specific set of primers and the length of the relative amplicons. All PCRs were performed using the following protocol: denaturation at 94°C for 30 sec; annealing at 55°C for 30 sec; extension at 72°C for 30 sec, with 35 cycles 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). Amplicons were visualized under UV light with a trans-illuminator and images acquired by a Canon digital camera. The analysis was repeated with two different replicates for each tissue sample. A semi-quantitative analysis of the expression of MSCs markers was performed evaluating the optical density of each positive band by means of the ImageJ image processing software normalized to the expression of the housekeeping gene GAPDH.

2.6.Statistical analysis

The results of the immunophenotypic characterization of MSC 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. All the tests were made using SPSS 16 IBM software.

3. Results

3.1.Cell culture and isolation

After 24 – 48 hours, cells were adherent to the surface of the flasks. Medium was changed after two-three days in order to eliminate non-adherent cells. During their growth, cells appeared as a homogeneous population of typical fibroblast-like cell morphology with elongated spindle like shape.

3.2.Tri-lineage cell differentiation

Cell cultures at P3 have were able to differentiate into osteogenic, adipogenic and chondrogenic lineages. A large number of cells contained intracellular lipid vacuoles stained with Oil Red O, confirming the adipogenic differentiation of sAT-MSC and pAT-MSC, following the appropriate stimulation in vitro. Cells stimulated with osteogenic induction medium formed aggregates and had numerous extracellular calcium deposits, as by with von Kossa staining confirmed the osteogenic differentiation of cells from both sources. The cell cultures treated with chondrogenic medium contained aggregates of proteoglycans stained in blue with Alcian Blue coloration. Control groups for each treatment showed no evidence of differentiation (Fig. 1).

3.3.FACS analysis

All antibodies tested on canine PBMC showed the expected reactivity (data not shown). For immunophenotypical analysis, the live population of MSC was gated in the scatter plot for further fluorescence intensity analysis in the histogram plot (Fig. 2).

Our data showed that MSC at P3 deriving from perivisceral and subcutaneous adipose tissue were positive for: CD29, CD44, CD73, CD90; and negative for CD34, CD45 and MHC-II

(Tables 3, 4). There was no significant difference in surface antigen expression between the two groups of MSC.

3.4.Reverse transcription analysis of gene expression

Expression analysis of a panel of genes consisting of CD90, CD73, CD105, CD45, CD34, CD44, CD13, CD29, CD31 and Oct-4 was performed on sAT-MSC and pAT-MSC at P3. Both populations were negative for CD45 and CD31. pAT-MSC were also negative for CD34 expression, while sAT-MSC showed weak expression. All other genes of the panel were expressed in both sAT-MSC and pAT-MSC. Semiquantitative analysis of marker profile, demonstrated a substantial equivalence between the two cell populations (Table 5).

4. Discussion

MSC-based treatment has been increasingly applied in veterinary medicine in the recent years. Several studies have reported the beneficial effects for different pathological states in dogs (Black et al., 2008, Hall et al., 2010, Pogue et al., 2013, Alamoudi et al., 2014, Marx et al., 2014, Penha et al., 2014, Villatoro et al., 2015, Perez-Merino et al., 2015, Kim et al., 2015 and Lee et al., 2015) , horses (Del Bue et al., 2008, Rich et al, 2014 and Govoni et al, 2015) and cats (Trzil, et al., 2015, Quimby, et al., 2016). 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 MSC and for the safety of clinical treatments based on these cells, it is necessary to have accurate cell characterization. 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.

Following the basic criteria for the characterization of human MSC (Dominici et al., 2006), equivalent MSC derived from laboratory and domestic animals were demonstrated to have fibroblast-like shape, ability to differentiate into osteogenic, chondrogenic and adipogenic lineages and have a determinate cell surface antigen pattern. However, the lack of species-specific antibodies has been a major drawback towards the full and accurate immunophenotypic characterization of animal derived MSC. Therefore, the first aim of our study was to select a panel of species-specific antibodies for the evaluation of cell surface CD markers expression in canine adipose tissue derived MSC that could be routinely used for the cell characterization prior to their use.

A further aim was to compare the marker expression of MSC derived from subcutaneous and perivisceral adipose tissue. Indeed, the characterization of these two populations of MSC would be of practical relevance for their clinical use. Subcutaneous adipose tissue is an easily available source of MSC. Samples of a few grams of fat tissue 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.

Cells 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. A previous study reported similar results (Guercio et al., 2013), but lacked the immunophenotypic characterization of the cell population. Thus, in the present study, we chose to determine the expression of a panel of cell surface CD markers (CD29, CD34, CD44, CD45, CD90, CD73 and MHC-II), typical for the MSC (Dominici et al., 2006) using anti-canine antibodies. CD 105 - one of the three surface markers that define human MSC (Dominici et al., 2006), was not taken in consideration in our study since we were not able to find any commercially available anti-CD105 canine antibodies.

Antibody reactivity was confirmed by flow cytometry of canine PBMC that served as a positive control. Interestingly, canine AT-MSC 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 MSC. RT-PCR analysis confirmed the results obtained with flow cytometry, as the two cell populations demonstrated a similar qualitative pattern of expression.

The comparison of the immunophenotypical profile of AT-MSC among canine, human and other species adipose tissue derived MSC would be of interest, as the dog is considered a suitable animal model for the study of human diseases.

In comparison to laboratory animals, canine anatomy and physiology, and the prevalence of natural occurring diseases with similar pathogenesis, more closely resemble humans. Dogs have a longer life-span, live in environments similar to humans and therefore are exposed to different external factors that are part of the aetiology of common diseases and conditions such as diabetes, obesity and tumors. The clinical follow-up is similar and there is a wide range of imaging techniques developed for veterinary patients (Volk and Theoret, 2013).

A complete overlap of marker expression between human MSC has yet to be demonstrated due to the lack of an extensive cell characterization in some species. However, similarities can be found between MSC 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. The identical expression of CD29, CD34, CD44, CD45, CD90 and MHC-II suggest that *in vitro* cultured canine MSC share most of their characteristics with human MSC. The unique difference found was in the expression of CD73, one of the three positive markers proposed for defining hMSC ($\geq$ 95%) (Dominici et al., 2006). Interestingly, we report here for the first time that the expression of CD73 is lower in an animal species compared to humans (Table 6). 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-MSC in veterinary patients.

Since bone marrow is commonly used in veterinary medicine for the isolation of MSC, we also compared our results with the immunophenotypical characteristics previously described for bone marrow derived MSC (BM-MSC) (Takemitsu et al., 2012) (Table 7). Both profiles are similar in terms of CD29, CD44 as positive markers and CD34, CD45 and MHC-II as negative markers. A difference exists in the expression of

CD73 and CD90, which appears to be lower in BM-MSC. However, the authors used non-canine specific antibodies for the analysis, which can have an influence on the interpretation of the percentage of expression. Altogether, the results presented in the table indicate a quite similar profile between canine adipose tissue and bone marrow derived MSC.

5. Conclusions

In conclusion, the present study reports the evaluation of a panel of 7 primary antibodies suitable for the flowcytometric characterization of canine MSC isolated from adipose tissue. Marker expression was the same in subcutaneous-derived and perivisceral-derived fat tissue. The substantial equivalence between the two sources of MSC has practical consequences in the clinical application of cell therapy, giving the veterinarian the opportunity to choose the source depending on the needs of each patient.

Conflict of interest statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Description of the panel of antibodies

Target cell marker	Clone	Reactivity	Production company
CD 29	TS2/16	Human;Dog; Bovine	BioLegend
CD 34	1H6	Dog	eBioscience
CD 44	YKIX337.8	Dog	eBioscience
CD 45	YKIX716.13	Dog	eBioscience
CD 73	Polyclonal	Human;Mouse;Rat; Dog; Chicken	Bioss
CD 90	YKIX337.217	Dog	eBioscience
MHC-II	YKIX337.8	Dog	eBioscience

Table 2. Markers used for the evaluation of cell surface markers expression of MSC by RT-PCR

Markers	Accession Number	Primers	Amplicon size
CD 13	NM_001146034.1	Fw: GGTCCTTACCATCACCTGGC Rv: CCTAAGGCCATCCATCGTCC	335bp
CD 29	XM_005616949.1	Fw: AGGATGTTGACGACTGCTGG Rv: ACCTTTGCATTCAGTGTTGTGC	356bp
CD 31	XM_848326	Fw: GCCCGAAGTTCACTCTCAAG Rv:CACTCCTTTGACCCACACCT	410bp
CD 34	NM_001003341.1	Fw: GAGATCACCCTAACGCCTGG Rv: GGCTCCTTCTCACACAGGAC	383bp
CD 44	NM_001197022.1	Fw: CCCATTACCAAAGACCACGA Rv: TTCTCGAGGTTCCGTGTCTC	408bp
CD 45	XM_005622282.1	Fw: TGTTTCCAGTTCTGTTTCCCCA Rv: TCAGGTACAAAGCCTTCCCA	432bp
CD 73	XM_532221.4	Fw: GATGGGAAAGGCAAGAGGCT Rv: TTCCTGGCATCTTGCTACGG	317bp
CD 90	NM_001287129.1	Fw: AAGCCAGGATTGGGGATGTG Rv: TGTGGCAGAGAAAGCTCCTG	285bp
CD 105	XM_005625330.2	Fw: GCTGAGGACAGAGATG	421bp

		ACCA Rv: CACGGAGGAGGAAGCT GAAG	
Oct-4	XM_538830.1	Fw: AAGCCTGCAGAAAGAC CTG Rv: GTTCGCTTTCTCTTTCG GGC	286bp

Table 3. Flow cytometric analysis of adipose tissue derived MSC

Markers	sAT-MSC [a]	pAT-MSC [b]
CD 29	99.5%±0.6	99.2%±1.5
CD 34	0.5%±1	0.25%±0.5
CD 44	78%±17	76.7%±18
CD 45	0.25%±0.5	0.5%±0.6
CD 73	14%±12.3	17%±14
CD 90	89%±6.7	79.5%±7.1
MHC-II	4.5%±6.3	4.5%±6.4

a and b: Data are displayed as percentages expressed as mean ± SD. Mood's median test was applied and no statistical difference for $P<0.05$ was found.

Table 4. Summary of cell surface markers expression measured by flow cytometry analysis

Cells	CD29	CD34	CD44	CD45	CD73	CD90	MHC-II
sAT-MSC	+++ [b]	- [c]	+++ [b]	- [c]	++ [a]	+++ [b]	- [c]
pAT-MSC	+++ [b]	- [c]	+++ [b]	- [c]	++ [a]	+++ [b]	- [c]

a ++ (10-40% positive cells)

b +++ (>40% positive cells)

c - (<5% positive cells).

Different groups are classified as described by Screven et al 2014

Table 5. Gene expression analysis of pAT-MSC and sAT-MSC using semi-quantitative RT-PCR

Cell marker	pAT-MSC	sAT-MSC
CD90	+++ [d]	+++ [d]
CD73	++ [c]	++ [c]
CD105	++ [c]	++ [c]
CD45	- [a]	- [a]
CD34	- [a]	+ [b]
CD44	+++ [d]	+++ [d]
CD13	+++ [d]	+++ [d]
CD29	+++ [d]	+++ [d]
CD31	- [a]	- [a]
Oct-4	+ [b]	+ [b]

a – (not expressed)

b + (<25% of GAPDH signal)

c ++ (25-50% of GAPDH signal)

d +++ (>50% of GAPDH signal)

Table 6. Comparison of the immunophenotypic profile of adipose derived MSC from multiple species

Species	Marker expression						
	CD 29	CD 34	CD 44	CD 45	CD 73	CD 90	MHC-II
Human[e]	+++ [c]	- [a]	+++ [c]	- [a]	+++ [c]	+++ [c]	- [a]
Mouse[f]	+++ [c]	/ [d]	+++ [c]	- [a]	++ [b]	+++ [c]	/ [d]
Rat[g]	+++ [c]	- [a]	/ [d]	- [a]	/ [d]	/ [d]	/ [d]
Dog[h]	+++ [c]	- [a]	+++ [c]	- [a]	++ [a]	+++ [c]	- [a]
Horse[i]	/ [d]	- [a]	+++ [c]	- [a]	- [a]	+++ [c]	- [a]
Cat[j]	/ [d]	- [a]	+++ [c]	- [a]	/ [d]	+++ [c]	/ [d]

a - (<5% positive cells)

b ++ (10-40% positive cells)

c +++ (>40% positive cells)

d / (not known)

Different groups are classified as described by Screven et al 2014

e (Dominici et al., 2006)

f (Taha and Hedayati, 2010) (Laschke et al., 2013)

g (Lotfy et al., 2014) (Tapp et al., 2008)

h *our results*

i (de Mattos Carvalho et al., 2009)(Pascucci et al., 2011)(Barberini et al., 2014)

j (Kono et al., 2014)

606 **Table 7 Comparison of the expression of the**
607 **immunophenotypic profile between MSC deriving from**
608 **subcutaneous adipose tissue, perivisceral adipose tissue and**
609 **bone marrow**

Markers	sAT-MSC	pAT-MSC	BM-MSC[a]
CD 29	99.5%±0.6	99.2%±1.5	98.41±0.53
CD34	0.5%±1	0.25%±0.5	0.88±0.21
CD 44	78%±17	76.7%±18	98.90±0.25
CD 45	0.25%±0.5	0.5%±0.6	0.24±0.07
CD 73	14%±12.3	17%±14	0.0081±0.01
CD 90	89%±6.7	79.5%±7.1	19.10±2.10
MHC-II	4.5%±6.3	4.5%±6.4	2.85±1.35

610 [a] Takemitsu et al., 2012

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Figure 1 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**) (40X, scale bar 200µm). 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**) (10X, scale bar 100µm). 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**) (10X, scale bar 100um).

Figure 2 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 (a) contains data regarding the MSC

deriving from subcutaneous adipose tissue and the low panel (b) data about MSC 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.

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Figure 1

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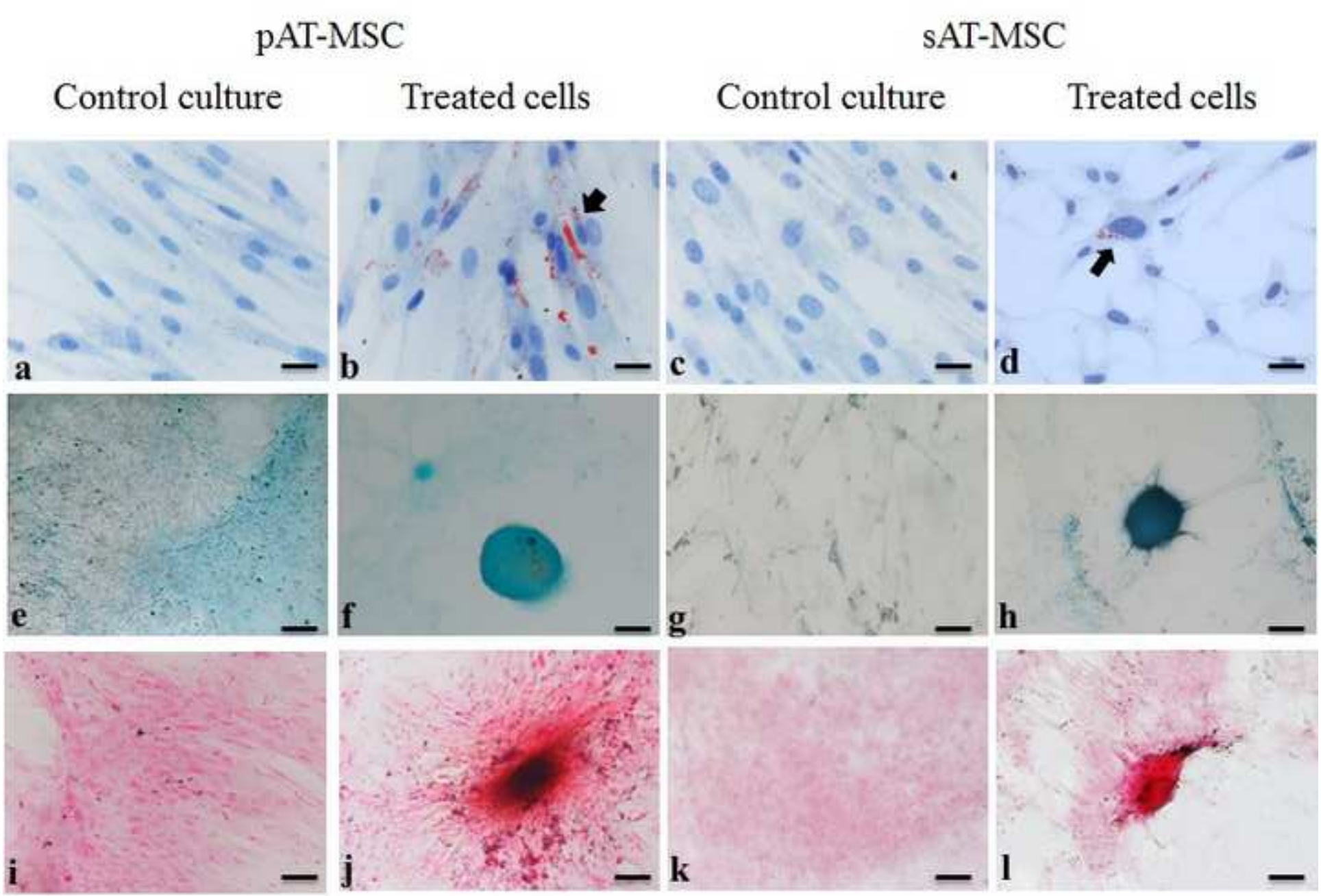

Figure 2

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