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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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1 Immunophenotypical characterization of canine mesenchymal
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22 **Abstract**

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28 mesenchymal stem cells (MSC). The aim of this study was to
29 select a panel of species-specific direct antibodies readily
30 useful for canine **mesenchymal stem cells MSC**
31 characterization. ~~MSC~~ **They were** isolated from perivisceral
32 (~~pAT-MSC~~) and subcutaneous (~~sAT-MSC~~) adipose tissue
33 samples collected during regular surgeries from 8 dogs.. Single
34 color flow cytometric analysis of **mesenchymal stem cells (P3)**
35 **deriving from subcutaneous and perivisceral adipose tissue**
36 ~~sAT-MSC and pAT-MSC (P3)~~ with a panel of 7 direct anti-
37 canine antibodies revealed two largely homogenous cell
38 populations with a similar pattern: CD29⁺, CD44⁺, CD73⁺,
39 CD90⁺, CD34⁻, CD45⁻ and MHC-II⁻ with no statistically
40 significant differences among them. **Antibody** ~~Antibodies~~
41 reactivity was demonstrated on canine peripheral blood
42 mononuclear cells. The similarities are reinforced by their *in*
43 *vitro* cell morphology, trilineage differentiation ability and RT-
44 PCR analysis (CD90⁺, CD73⁺, CD105⁺, CD44⁺, CD13⁺,
45 CD29⁺, Oct-4⁺ gene and CD31⁻ and CD45⁻ expression). Our
46 results report for the first time a comparison between the

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

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

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68 **1. Introduction**

69 Regenerative medicine and tissue engineering are promising
70 novel therapeutic approaches in veterinary medicine. In
71 particular, mesenchymal stem cells (MSC) are ~~the most~~
72 commonly used as a ~~option as a~~ therapeutic tool within the field
73 of cell based therapies and have been the subject of a number of
74 preclinical and clinical studies. Mesenchymal stem cells MSC
75 are self-renewing, multipotent adult stem cells found near
76 blood vessels in different organs such as bone marrow, adipose
77 tissue, blood, umbilical cord, muscle, bone and cartilage. Due
78 to their ability to differentiate into other cell types and to
79 produce a wide range of immunomodulatory, trophic,
80 angiogenic and anti-apoptotic bioactive molecules, MSC have a
81 practical application in the field of cell therapy (Marx et al.,
82 2015). The Mesenchymal and Tissue Stem Cell Committee of
83 the International Society for Cellular Therapy defined them as a
84 population of cells that satisfies the following criteria in vitro:
85 1) adherence to a plastic surface and typical spindle-like shape;
86 2) ability to differentiate in osteogenic, adipogenic and
87 chondrogenic lineage and 3) the expression of a set of cell
88 surface markers (Dominici et al., 2006).

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

91 The expression of cell surface markers on MSC deriving from
92 veterinary species is subject of intense research at the moment.

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

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

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

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

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

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

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

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

143 **2. Materials and methods**

144 *2.1. Study design*

145 Eight client owned canine patients from different breeds, 5
146 females and 3 males, weighing from 4 to 60 kg, aged between
147 1,5 and 12 years were selected for adipose tissue sampling
148 during scheduled surgical procedures (Table 1). Patients
149 underwent routine clinical, biochemical and haematological
150 examination prior the surgical procedure. All animal
151 procedures and protocols were performed by licensed
152 veterinary surgeons under standard ethical and sterile
153 conditions. The owners signed an informed consent and agreed
154 on the participation of their dogs.

155

156 *2.2. Sample collection*

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

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

163 (0.10U/ml), streptomycin (0,10 µg/ml) and amphotericin B (2,5
164 µg/ml, Gibco) until cell isolation (not more than 4 hours).
165 Peripheral blood samples for the isolation and flow cytometry
166 analysis of peripheral blood mononuclear cells were collected
167 from 3 healthy dogs prior to orthopaedic surgery. All the
168 samples were collected following owner consent, under
169 standard ethical and sterile conditions.

170 *2.3. Cell culture*

171 MSC from subcutaneous adipose tissue (sAT-MSC) and
172 perivisceral adipose tissue (pAT-MSC) were isolated from
173 adipose tissue samples weighing 2 grams. The samples were
174 cut with scalpels in small pieces of 0,5 cm diameter and
175 afterwards were transferred in a 15mL Conical Centrifuge
176 *Falcon* Tube containing 10 ml of collagenase solution (DMEM
177 low glucose, Gibco; penicillin 0,10 U/ml, Gibco; streptomycin
178 0,10µg/ml, Gibco; amphotericin B 2,5 µg/ml, Gibco;
179 collagenase type I 0,1% p/v). Enzymatic digestion was
180 performed under a mechanical stirrer at 37°C for 45min.

181 The digested sample was then centrifuged (210xg for 8 min).
182 After removal of the collagenase solution and the fat
183 supernatant, the cell pellet was resuspended in 500µl
184 maintenance medium (DMEM low glucose, Gibco added with
185 10% fetal bovine serum (FBS), Gibco; penicillin 0,10U/ml,
186 Gibco; streptomycin 0,10µg, Gibco; amfotericine B 2,5 µg/ml,

187 Gibco) and seeded in 25 cm² (Orange Scientific Tissue Culture
188 Flasks) containing 5 ml of DMEM. The cells were maintained
189 in an incubator at 37°C at 5% CO₂. The medium was changed
190 each 2-3 days. Cells were cultured to 80% confluence and then
191 trypsinized with 0,05% Trypsin-EDTA solution (Gibco) The
192 cell culture was grown until passage 3 (P3) when cells were
193 used for the evaluation of the differentiation capacity, flow
194 cytometric analysis and RT-PCR.

195 *2.4.Tri-lineage cell differentiation*

196 1. Adipogenic differentiation

197 MSC deriving from subcutaneous and perivisceral adipose
198 tissue, at P3 were seeded in six-well plates at a density of 6 x
199 10³ cells/cm². In each well, 3 ml of DMEM was added and the
200 cells were incubated at 37°C and 5% CO₂. At a confluency of
201 80% they were treated with adipogenic differentiation media
202 (StemPro Adipogenesis Differentiation Kit). The flasks were
203 then put in an incubator with humidified atmosphere of 5%
204 CO₂ and temperature of 37°C. Medium was changed each 2-3
205 days. After 21 days the cells were fixed with 70% ethanol and
206 stained with Oil Red O coloration.

207 2. Chondrogenic differentiation

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

210 of 6×10^3 cells/cm². In each well 3 ml of DMEM was added
211 and the cells were incubated at 37°C and 5% CO₂. At a
212 confluency of 80% they were treated with with chondrogenic
213 differentiation medium (StemPro Chondrogenesis
214 Differentiation Kit). The flasks were then put in an incubator
215 with humidified atmosphere of 5% CO₂ and temperature of 37
216 °C. Medium was changed each 2-3 days. After 21 days the cells
217 were fixed with 4% formaldehyde and stained with Alcian blue.

218 3. Osteogenic differentiation

219 MSC deriving from subcutaneous and perivisceral adipose
220 tissue, at passage 3 were seeded in six-well plates at a density
221 of 6×10^3 cells/cm². In each well 3 ml of DMEM was added
222 and the cells were incubated at 37°C and 5% CO₂. Arriving at
223 confluency of 80%, the cells were treated with osteogenic
224 induction medium (DMEM additioned with 100nM
225 dexamethasone, 10µM glycerophosphate and 0.250 mM
226 ascorbic acid). Medium was changed each 2-3 days. After 21
227 days the cells were fixed with 1% paraformaldehyde and
228 stained with von Kossa staining (Bio Optica).

229 2.5. Immunophenotyping

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

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

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

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

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

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

257 1077 solution (Sigma, St. Louis, MO) according to the
258 manufacturer's instructions. Blood samples were taken and
259 then stratified on Histopaque-1077 solution (1:1, v/v, Sigma)
260 and centrifuged at $400 \times g$ for 30 min; purified PBMC were
261 washed with sterile Phosphate Buffer Solution (PBS) (Sigma)
262 supplemented with 1% fetal FBS and resuspended in RPMI-
263 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10%
264 FBS, 2 mM l-glutamine, 100mM non-essential amino-acids, 50
265 mM 2-mercaptoethanol (Sigma) and 100 U/ml penicillin G,
266 100 $\mu\text{g/ml}$ streptomycin and 0.25 $\mu\text{g/ml}$ amphotericin B.

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

276 *2.6.RT-PCR*

277 Assessment of the phenotypic expression of different cell
278 markers was performed by Reverse-Transcriptase-PCR (RT-
279 PCR). Total RNA was extracted from 1.5×10^6 cells at P3 (80%
280 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 MgCl₂,
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*

306 The results of the immunophenotypic characterization of MSC
307 deriving from perivisceral and subcutaneous adipose tissue
308 were compared. Data was expressed as mean± standard
309 deviation (SD). The differences among the groups were
310 considered statistically significant for P<0.05 and Mood's
311 median test was made as non-parametric statistics analysis. All
312 the tests were made using SPSS 16 IBM software.

313 **3. Results**

314 *3.1. Cell culture and isolation*

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

320 *3.2. Tri-lineage cell differentiation*

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

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

334 *3.3.FACS analysis*

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

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

345 *3.4.Reverse transcription analysis of gene expression*

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

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

355 **4. Discussion**

356 MSC-based treatment has been increasingly applied in
357 veterinary medicine in the recent years. Several studies have
358 reported the beneficial effects for different pathological states
359 in dogs (Black et al., 2008, Hall et al., 2010, Pogue et al., 2013,
360 Alamoudi et al., 2014, Marx et al., 2014, Penha et al., 2014,
361 Villatoro et al., 2015, Perez-Merino et al., 2015, Kim et al.,
362 2015 and Lee et al., 2015). ~~horses (Del Bue et al., 2008, Rieh~~
363 ~~et al., 2014 and Govoni et al., 2015) and cats (Trzil, et al., 2015,~~
364 ~~Quimby, et al., 2016).~~ Although the majority of the studies
365 carried out contain a limited number of patients and often lack
366 of appropriate controls, the results can be considered
367 encouraging for the set-up of clinical protocols to be applied in
368 veterinary medicine.

369 For the reliability of research studies dealing with biological
370 properties of MSC and for the safety of clinical treatments
371 based on these cells, it is necessary to have accurate cell
372 characterization. The availability of several possible tissue
373 sources, different methods of cell culture, expansion and
374 handling, as well as a variety of possible therapeutic

375 approaches, represent a limit to the correct evaluation of the
376 healing potential of these cells.

377 Following the basic criteria for the characterization of human
378 MSC (Dominici et al., 2006), equivalent MSC derived from
379 laboratory and domestic animals were demonstrated to have
380 fibroblast-like shape, ability to differentiate into osteogenic,
381 chondrogenic and adipogenic lineages and to have a
382 determinate cell surface antigen pattern. ~~However, the lack of~~
383 ~~species-specific antibodies has been a major drawback towards~~
384 ~~the full and accurate immunophenotypic characterization of~~
385 ~~animal-derived MSC.~~ Therefore, The first aim of our study was
386 to select a panel of species-specific antibodies for the
387 evaluation of cell surface CD ~~marker markers~~ expression in
388 canine adipose tissue derived MSC that could be routinely used
389 for the cell characterization prior to their use. **Having read**
390 **previous studies addressing the same topic, we encountered a**
391 **common statement declared by researchers, saying that their**
392 **findings can be found unexpected or contradictory due to the**
393 **low binding affinity of non species-specific antibodies used for**
394 **the screening of cell surface markers (Screven et al., 2014).**
395 **We hypothesized that by using a panel of species-specific**
396 **antibodies this variable would be eliminated. The panel would**
397 **give more reliable and reproducible results that are necessary**
398 **for the correct characterization of MSC, since they have**

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

401 A further aim was to compare the marker expression of MSC
402 derived from subcutaneous and perivisceral adipose tissue.
403 Indeed, the characterization of these two populations of MSC
404 would be of practical relevance for their clinical use.

405 Subcutaneous adipose tissue is an easily available source of
406 MSC. Samples of a few grams of fat tissue can be collected
407 quickly and safely during surgery in dogs of different sizes,
408 avoiding the potential risk associated with abdominal surgery
409 or bone marrow aspiration. We hypothesized that by defining
410 and comparing the cell surface pattern of subcutaneous and
411 perivisceral adipose tissue, we could obtain data that will offer
412 researchers and veterinary practitioners the opportunity to
413 choose the source of MSC for their applications.

414

415 MSC derived from the two tissue sources were analysed at P3,
416 as this passage is considered appropriate for obtaining an
417 adequate number of cells, safe in terms of chromosome
418 variability and genetic abnormalities, and, therefore, adequate
419 for therapeutic applications (Binato et al., 2013). Cells-MSC
420 from both tissue sources had a similar fibroblast-like
421 morphology, were able to adhere to plastic surface, grew in
422 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-II⁻. As far as**
447 **we know, this is the first study that characterized cAT-MSC by**

448 using a panel of canine species specific antibodies for both
449 positive as well as negative markers. We are aware that culture
450 conditions influence the immunophenotypic profile of MSC, as
451 it has been demonstrated for example, by bFGF (Gharibi and
452 Hughes, 2012). Therefore for each future characterization in
453 different culture conditions it is recommended to take this fact
454 in consideration. Different researchers included anti-human
455 antibodies in their protocols, however, they cannot be
456 considered fully reliable for the characterization of canine
457 MSC. Rozemuller et al., 2010 studied the expression of surface
458 cell surface marker expression on canine BM-MSC using a
459 panel of 43 anti-human antibodies. 24 of them, among which
460 CD73, CD90 and CD105 did react with human MSC, but failed
461 to cross-react with canine MSC, therefore cannot be considered
462 adequate for the definition of the immune phenotype of canine
463 MSC.. Takemitsu et al., isolated and characterized canine AT-
464 MSC obtaining surprisingly low results for the expression of
465 CD90 and CD105, finding that can be justified by the fact that
466 they used non canine specific antibodies. Similar findings were
467 obtained by Screven et al. for CD105, as they stated that the
468 low binding activity can be a consequence to the use of a
469 nonspecific canine antibody.

470 By RT-PCR analysis, we analyzed the gene expression of the
471 same markers analyzed with FACS analysis, including
472 additionally Oct-4, CD105, CD13 and CD31. The positive

473 expression of Oct-4 demonstrated the pluripotency of both
474 sAT-MSC as well as pAT-MSC, as it has already been
475 described in canine (Neupane et al., 2008, Reich et al., 2012)
476 and human (Han et al., 2014) AT-MSC. Furthermore, the cells
477 resulted positive for CD13 and negative for CD 31. Although
478 we did not evaluate CD105 expression by flow cytometry
479 analysis, the RT-PCR revealed a positive expression for both
480 sAT-MSC and pAT-MSC. As a whole the results obtained by
481 flow cytometry were confirmed by RT-PCR, as the two cell
482 populations demonstrated a similar qualitative pattern of
483 expression, with the only exception of CD34. According to
484 Takemitsu et al. (2012), and Screven et al. (2014), canine AT-MSC
485 do not express CD34. However Russell et al. (2016) found a
486 moderately high expression of CD34 on AT-MSC, explaining that
487 actually the fat harvesting site does have an influence on surface
488 markers expression. We indeed detected a positive, although low
489 expression of CD34 at mRNA level in sAT-MSC. On the other hand
490 at a protein level CD34 was absent. This finding could be explained
491 with the observation that sometimes low level of gene transcription
492 does not result in a significant protein synthesis (Screven et al.,
493 2014).

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496 The comparison of the immunophenotypical profile of AT-
497 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 ($\geq 95\%$)
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. Takemitsu 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**

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

558 **5. Conclusions**

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

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

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579 **Conflict of interest statement**

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595 **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

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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_00114 6034.1	Fw: GGTCCTTACCATCACCTG GC Rv: CCTAAGGCCATCCATCGT CC	335bp
CD 29	XM_00561 6949.1	Fw: AGGATGTTGACGACTGCT GG Rv: ACCTTTGCATTCAGTGTT GTGC	356bp
CD 31	XM_84832 6	Fw: GCCCGAAGTTCACTCTCA AG Rv:CACTCCTTTGACCCAC ACCT	410bp
CD 34	NM_00100 3341.1	Fw: GAGATCACCCCTAACGCCT GG Rv: GGCTCCTTCTCACACAGG AC	383bp
CD 44	NM_00119 7022.1	Fw: CCCATTACCAAAGACCAC GA Rv: TTCTCGAGGTTCCGTGTC TC	408bp
CD 45	XM_00562 2282.1	Fw: TGTTTCCAGTTCTGTTTCC CCA Rv: TCAGGTACAAAGCCTTCC CA	432bp
CD 73	XM_53222 1.4	Fw: GATGGGAAAGGCAAGAG GCT Rv: TTCCTGGCATCTTGCTAC GG	317bp
CD 90	NM_00128 7129.1	Fw: AAGCCAGGATTGGGGAT GTG Rv: TGTGGCAGAGAAAGCTCC TG	285bp
CD 105	XM_00562 5330.2	Fw: GCTGAGGACAGAGATGA	421bp

Oct-4	XM_53883	CCA	
	0.1	Rv:	
		CACGGAGGAGGAAGCTG	
		AAG	
		Fw:	286bp
		AAGCCTGCAGAAAGACCT	
		G	
		Rv:	
		GTTTCGCTTTCTCTTTTCGGG	
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651 **Table 4. Flow cytometric analysis of adipose tissue derived**
 652 **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

653 a and b: Data are displayed as percentages expressed as mean ± SD. Mood's median

654 test was applied and no statistical difference for P<0.05 was found.

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674 **Table 5. Summary of cell surface markers expression**
 675 **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

676 **a** ++ (10-40% positive cells)

677 **b** +++ (>40% positive cells)

678 **c** - (<5% positive cells).

679 Different groups are classified as described by Screven et al 2014

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695 **Table 6. Gene expression analysis of pAT-MSC and sAT-**
 696 **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

697 **a** – (not expressed)
 698 **b** + (<25% of GAPDH signal)
 699 **c** ++ (25-50% of GAPDH signal)
 700 **d** +++ (>50% of GAPDH signal)
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720 **Table 7. Comparison of the immunophenotypic profile of**
 721 **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
Horseⁱ	/ ^d	- ^a	+++ ^c	- ^a	- ^a	+++ ^c	- ^a
Cat^j	/ ^d	- ^a	+++ ^c	- ^a	/ ^d	+++ ^c	/ ^d

722 **a** - (<5% positive cells)

723 **b** ++ (10-40% positive cells)

724 **c** +++ (>40% positive cells)

725 **d** / (not known)

726 Different groups are classified as described by Screven et al 2014

727 **e** (Dominici et al., 2006)

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

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

730 **h** *our results*

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

732 **j** (Kono et al., 2014)

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747 **Table 8. Comparison of the expression of the**
 748 **immunophenotypic profile between MSC deriving from**
 749 **subcutaneous adipose tissue, perivisceral adipose tissue and**
 750 **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

751 ^aTakemitsu et al., 2012

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767 **Figure 1** In vitro tri-lineage cell differentiation

768 Photomicrographs of canine perivisceral adipose tissue derived
769 MSC- (pAT-MSC: **b, f, j**) and canine subcutaneous adipose
770 tissue derived MSC (sAT-MSC: **d, g, l**), after 21 days of
771 culture in adipogenic (**b, d**), chondrogenic (**f, h**), osteogenic
772 (**j,l**) induction medium. Parallely a control culture of pAT-MSC
773 (**a, e, i**) and sAT-MSC (**c, g, k**) was grown for 21 days in
774 DMEM. Adipogenic differentiation is evidenced with the
775 presence of intracellular vacuoles colored in red (black arrow)
776 with Oil Red O staining, in pAT-MSC (**b**) and sAT- MSC (**d**),
777 which are absent in control groups (**a,c**) (40X, scale bar
778 200µm). Alcian blue staining indicated the presence of
779 aggregates of proteglycans present in treated pAT-MSC (**f**) and
780 sAT-MSC (**h**), and their absence in the control cultures (**e,h**)
781 (10X, scale bar 100µm). Osteogenic differentiation was
782 indicated with extracellular calcium aggregates stained with
783 von Kossa coloration in pAT-MSC (**j**) and sAT-MSC (**l**)
784 stimulated with ostegenic medium. Control groups did not
785 contain any calcium aggregates (**i, k**) (10X, scale bar 100um).

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789 **Figure 2** Flow cytometry analysis of the expression of cell
790 surface markers on sAT-MSC and pAT-MSC

791 The expression of cell surface markers is presented in two
792 panels. The upper panel (a) contains data regarding the MSC
793 deriving from subcutaneous adipose tissue and the low panel
794 (b) data about MSC deriving from perivisceral adipose tissue.
795 Live cell populations were gated in forward and side scatter for
796 further analysis. Each histogram contains two peaks. The
797 shaded red peak represents the actual expression of the markers
798 for each cell group, on the other hand the transparent one is the
799 negative control.

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- 1 Highlights
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- 3 • The design of MSC based therapeutic protocols requires an
- 4 accurate cell characterization
- 5 • Selection of a panel of 7 species specific anti-
- 6 canine antibodies for flow cytometric
- 7 characterization of canine AT-MSC
- 8 • Canine AT-MSC from subcutaneous and perivisceral
- 9 adipose tissue have a similar
- 10 immunophenotypic profile
- 11 • Comparison of the immunophenotypical profile of
- 12 AT-MSC from veterinary species,
- 13 laboratory animals and human origin
- 14 • AT-MSC derived from animal species have a lower CD73
- 15 expression compared to human
- 16 AT-MSC

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

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22 **Abstract**

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

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

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

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67 **1. Introduction**

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

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

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

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

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

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

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

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

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

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

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

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138 **2. Materials and methods**

139 *2.1. Sample collection*

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

143 After collection each sample was transferred to a vial
144 containing 20 ml of Dulbecco's Eagle Modified Medium
145 (DMEM low glucose, Gibco) additioned with penicillin
146 (0,10U/ml), streptomycin (0,10 µg/ml) and amphotericin B (2,5
147 µg/ml, Gibco) until cell isolation (not more than 4 hours).

148 Peripheral blood samples for the isolation and flow cytometry
149 analysis of peripheral blood mononuclear cells were collected
150 from 3 healthy dogs prior to orthopaedic surgery. All the
151 samples were collected following owner consent, under
152 standard ethical and sterile conditions.

153 *2.2. Cell culture*

154 MSC from subcutaneous adipose tissue (sAT-MSC) and
155 perivisceral adipose tissue (pAT-MSC) were isolated from
156 adipose tissue samples weighing 2 grams. The samples were
157 cut with scalpels in small pieces of 0,5 cm diameter and
158 afterwards were transferred in a 15mL Conical Centrifuge
159 *Falcon* Tube containing 10 ml of collagenase solution (DMEM
160 low glucose, Gibco; penicillin 0,10 U/ml, Gibco; streptomycin

161 0,10µg/ml, Gibco; amphotericin B 2,5 µg/ml, Gibco;
162 collagenase type I 0,1% p/v). Enzymatic digestion was
163 performed under a mechanical stirrer at 37°C for 45min.

164 The digested sample was then centrifuged (210xg for 8 min).
165 After removal of the collagenase solution and the fat
166 supernatant, the cell pellet was resuspended in 500µl
167 maintenance medium (DMEM low glucose, Gibco added with
168 10% fetal bovine serum (FBS), Gibco; penicillin 0,10U/ml,
169 Gibco; streptomycin 0,10µg, Gibco; amfotericine B 2,5 µg/ml,
170 Gibco) and seeded in 25 cm² (Orange Scientific Tissue Culture
171 Flasks) containing 5 ml of DMEM. The cells were maintained
172 in an incubator at 37°C at 5% CO₂. The medium was changed
173 each 2-3 days. Cells were cultured to 80% confluence and then
174 trypsinized with 0,05% Trypsin-EDTA solution (Gibco) The
175 cell culture was grown until passage 3 (P3) when cells were
176 used for the evaluation of the differentiation capacity, flow
177 cytometric analysis and RT-PCR.

178 *2.3.Tri-lineage cell differentiation*

179 1. Adipogenic differentiation

180 MSC deriving from subcutaneous and perivisceral adipose
181 tissue, at P3 were seeded in six-well plates at a density of 6 x
182 10³ cells/cm². In each well, 3 ml of DMEM was added and the
183 cells were incubated at 37°C and 5% CO₂. At a confluency of
184 80% they were treated with adipogenic differentiation media

185 (StemPro Adipogenesis Differentiation Kit). The flasks were
186 then put in an incubator with humidified atmosphere of 5%
187 CO₂ and temperature of 37°C. Medium was changed each 2-3
188 days. After 21 days the cells were fixed with 70% ethanol and
189 stained with Oil Red O coloration.

190 2. Chondrogenic differentiation

191 MSC deriving from subcutaneous and perivisceral adipose
192 tissue, at passage 3 were seeded in six-well plates at a density
193 of 6×10^3 cells/cm². In each well 3 ml of DMEM was added
194 and the cells were incubated at 37°C and 5% CO₂. At a
195 confluency of 80% they were treated with with chondrogenic
196 differentiation medium (StemPro Chondrogenesis
197 Differentiation Kit). The flasks were then put in an incubator
198 with humidified atmosphere of 5% CO₂ and temperature of 37
199 °C. Medium was changed each 2-3 days. After 21 days the cells
200 were fixed with 4% formaldehyde and stained with Alcian blue.

201 3. Osteogenic differentiation

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

209 ascorbic acid). Medium was changed each 2-3 days. After 21
210 days the cells were fixed with 1% paraformaldehyde and
211 stained with von Kossa staining (Bio Optica).

212 *2.4.Immunophenotyping*

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

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

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

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

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

235 Antibodies were tested on peripheral blood mononuclear cells
236 (PBMC) of 3 healthy dogs to confirm reactivity. PBMC were
237 isolated from 2 ml of 3 dog blood samples collected in lithium-
238 heparin as anticoagulant by density gradient in Histopaque-
239 1077 solution (Sigma, St. Louis, MO) according to the
240 manufacturer's instructions. Blood samples were taken and
241 then stratified on Histopaque-1077 solution (1:1, v/v, Sigma)
242 and centrifuged at $400 \times g$ for 30 min; purified PBMC were
243 washed with sterile Phosphate Buffer Solution (PBS) (Sigma)
244 supplemented with 1% fetal bovine serum (FBS) and
245 resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA)
246 supplemented with 10% FBS, 2 mM l-glutamine, 100mM non-
247 essential amino-acids, 50 mM 2-mercaptoethanol (Sigma) and
248 100 U/ml penicillin G, 100 μ g/ml streptomycin and 0.25 μ g/ml
249 amphotericin B.

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

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

259 *2.5.RT-PCR*

260 Assessment of the phenotypic expression of different cell
261 markers was performed by Reverse-Transcriptase-PCR (RT-
262 PCR). Total RNA was extracted from 1.5×10^6 cells at P3 (80%
263 of confluence) using the kit Nucleospin® RNA II (Macherey-
264 Nagel) following manufacturer's instructions. cDNA was then
265 obtained via reverse transcription of 1.5µg of total RNA using
266 RevertAit™ First Strand cDNA Synthesis Kit (Fermentas).
267 PCR was performed using 2µl of cDNA. The final PCR
268 mixture contained 1x amplification buffer with 2.5 mM MgCl₂,
269 10mM dNTP Mix (Thermo Scientific), 0,25µm specific
270 forward and reverse primers, 1 U Dream Taq (Thermo
271 Scientific) in a final volume of 25µl.

272 Table 2 reports the list of genes whose expression was
273 analyzed, their gene accession number, the sequence of forward
274 and reverse specific set of primers and the length of the relative
275 amplicons. All PCRs were performed using the following
276 protocol: denaturation at 94°C for 30 sec; annealing at 55°C for
277 30 sec; extension at 72°C for 30 sec, with 35 cycles The
278 products of the RT-PCR were separated on agarose gel (1,5%
279 P/V) in TAE buffer stained with 3,5µl ethidium bromide

280 (10mg/ml). Amplicons were visualized under UV light with a
281 trans-illuminator and images acquired by a Canon digital
282 camera. The analysis was repeated with two different replicates
283 for each tissue sample. A semi-quantitative analysis of the
284 expression of MSCs markers was performed evaluating the
285 optical density of each positive band by means of the ImageJ
286 image processing software normalized to the expression of the
287 housekeeping gene GAPDH.

288 *2.6. Statistical analysis*

289 The results of the immunophenotypic characterization of MSC
290 deriving from perivisceral and subcutaneous adipose tissue
291 were compared. Data was expressed as mean± standard
292 deviation (SD). The differences among the groups were
293 considered statistically significant for $P < 0.05$ and Mood's
294 median test was made as non-parametric statistics analysis. All
295 the tests were made using SPSS 16 IBM software.

296 **3. Results**

297 *3.1. Cell culture and isolation*

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

303 *3.2.Tri-lineage cell differentiation*

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

317 *3.3.FACS analysis*

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

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

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

328 *3.4. Reverse transcription analysis of gene expression*

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

338 **4. Discussion**

339 MSC-based treatment has been increasingly applied in
340 veterinary medicine in the recent years. Several studies have
341 reported the beneficial effects for different pathological states
342 in dogs (Black et al., 2008, Hall et al., 2010, Pogue et al., 2013,
343 Alamoudi et al., 2014, Marx et al., 2014, Penha et al., 2014,
344 Villatoro et al., 2015, Perez-Merino et al., 2015, Kim et al.,
345 2015 and Lee et al., 2015) , horses (Del Bue et al., 2008, Rich
346 et al, 2014 and Govoni et al, 2015) and cats (Trzil, et al., 2015,
347 Quimby, et al., 2016). Although the majority of the studies
348 carried out contain a limited number of patients and often lack
349 of appropriate controls, the results can be considered

350 encouraging for the set-up of clinical protocols to be applied in
351 veterinary medicine.

352 For the reliability of research studies dealing with biological
353 properties of MSC and for the safety of clinical treatments
354 based on these cells, it is necessary to have accurate cell
355 characterization. The availability of several possible tissue
356 sources, different methods of cell culture, expansion and
357 handling, as well as a variety of possible therapeutic
358 approaches, represent a limit to the correct evaluation of the
359 healing potential of these cells.

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

373 A further aim was to compare the marker expression of MSC
374 derived from subcutaneous and perivisceral adipose tissue.
375 Indeed, the characterization of these two populations of MSC
376 would be of practical relevance for their clinical use.
377 Subcutaneous adipose tissue is an easily available source of
378 MSC. Samples of a few grams of fat tissue can be collected
379 quickly and safely during surgery in dogs of different sizes,
380 avoiding the potential risk associated with abdominal surgery
381 or bone marrow aspiration.

382 Cells from both tissue sources had a similar fibroblast-like
383 morphology, were able to adhere to plastic surface, grew in
384 monolayer and demonstrated the capacity to differentiate in
385 osteogenic, chondrogenic and adipogenic lineages when
386 stimulated with appropriate induction medium. A previous
387 study reported similar results (Guercio et al., 2013), but lacked
388 the immunophenotypic characterization of the cell population.
389 Thus, in the present study, we chose to determine the
390 expression of a panel of cell surface CD markers (CD29, CD34,
391 CD44, CD45, CD90, CD73 and MHC-II), typical for the MSC
392 (Dominici et al., 2006) using anti-canine antibodies. CD 105 -
393 one of the three surface markers that define human MSC
394 (Dominici et al., 2006), was not taken in consideration in our
395 study since we were not able to find any commercially
396 available anti-CD105 canine antibodies.

397 Antibody reactivity was confirmed by flow cytometry of canine
398 PBMC that served as a positive control. Interestingly, canine
399 AT-MSC derived from subcutaneous and perivisceral adipose
400 tissue showed a similar immunophenotypic pattern, which from
401 a practical point of view indicates that both tissues can be used
402 as a valid source for the isolation of MSC. RT-PCR analysis
403 confirmed the results obtained with flow cytometry, as the two
404 cell populations demonstrated a similar qualitative pattern of
405 expression.

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

410 In comparison to laboratory animals, canine anatomy and
411 physiology, and the prevalence of natural occurring diseases
412 with similar pathogenesis, more closely resemble humans.
413 Dogs have a longer life-span, live in environments similar to
414 humans and therefore are exposed to different external factors
415 that are part of the aetiology of common diseases and
416 conditions such as diabetes, obesity and tumors. The clinical
417 follow-up is similar and there is a wide range of imaging
418 techniques developed for veterinary patients (Volk and Theoret,
419 2013).

420 A complete overlap of marker expression between human MSC
421 has yet to be demonstrated due to the lack of an extensive cell
422 characterization in some species. However, similarities can be
423 found between MSC derived from domestic and laboratory
424 animals and humans. Most of the markers analyzed in this
425 study share a similar distribution in the different species, with
426 the exception of CD73. The identical expression of CD29,
427 CD34, CD44, CD45, CD90 and MHC-II suggest that *in vitro*
428 cultured canine MSC share most of their characteristics with
429 human MSC. The unique difference found was in the
430 expression of CD73, one of the three positive markers proposed
431 for defining hMSC ($\geq 95\%$) (Dominici et al., 2006).
432 Interestingly, we report here for the first time that the
433 expression of CD73 is lower in an animal species compared to
434 humans (Table 6). This would suggest that there is a slightly
435 different immunophenotypic profile that should be taken in
436 consideration for the correct interpretation and characterization
437 of AT-MSc in veterinary patients.

438 Since bone marrow is commonly used in veterinary medicine
439 for the isolation of MSC, we also compared our results with the
440 immunophenotypical characteristics previously described for
441 bone marrow derived MSC (BM-MSc) (Takemitsu et al.,
442 2012) (Table 7). Both profiles are similar in terms of CD29,
443 CD44 as positive markers and CD34, CD45 and MHC-II as
444 negative markers. A difference exists in the expression of

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

451 **5. Conclusions**

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

461 **Conflict of interest statement**

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463 agencies in the public, commercial, or not-for-profit sectors.

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466 Veterinary Clinic “Jenner”, Parma, led by Dr. Emanuele
467 Cabibbo for the help in collecting adipose tissue samples.

468 **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; Chicken	Dog; Bioss
CD 90	YKIX337.217	Dog	eBioscience
MHC-II	YKIX337.8	Dog	eBioscience

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

Markers	Accession Number	Primers	Amplicon size
CD 13	NM_0011 46034.1	Fw: GGTCCTTACCATCACCT GGC Rv: CCTAAGGCCATCCATC GTCC	335bp
CD 29	XM_0056 16949.1	Fw: AGGATGTTGACGACTG CTGG Rv: ACCTTTGCATTTCAGTGT TGTGC	356bp
CD 31	XM_8483 26	Fw: GCCCGAAGTTCACCTCTC AAG Rv:CACTCCTTTGACCCA CACCT	410bp
CD 34	NM_0010 03341.1	Fw: GAGATCACCCCTAACGC CTGG Rv: GGCTCCTTCTCACACAG GAC	383bp
CD 44	NM_0011 97022.1	Fw: CCCATTACCAAAGACC ACGA Rv: TTCTCGAGGTTCCGTGT CTC	408bp
CD 45	XM_0056 22282.1	Fw: TGTTTCCAGTTCTGTTT CCCCA Rv: TCAGGTACAAAGCCTT CCCA	432bp
CD 73	XM_5322 21.4	Fw: GATGGGAAAGGCAAGA GGCT Rv: TTCCTGGCATCTTGCTA CGG	317bp
CD 90	NM_0012 87129.1	Fw: AAGCCAGGATTGGGGA TGTG Rv: TGTGGCAGAGAAAGCT CCTG	285bp
CD 105	XM_0056 25330.2	Fw: GCTGAGGACAGAGATG	421bp

		ACCA	
		Rv:	
		CACGGAGGAGGAAGCT	
		GAAG	
Oct-4	XM_5388	Fw:	286bp
	30.1	AAGCCTGCAGAAAGAC	
		CTG	
		Rv:	
		GTTCGCTTTCTCTTTCG	
		GGC	

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511 **Table 3. Flow cytometric analysis of adipose tissue derived**
 512 **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

513 a and b: Data are displayed as percentages expressed as mean ± SD. Mood's median

514 test was applied and no statistical difference for P<0.05 was found.

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535 **Table 4. Summary of cell surface markers expression**
 536 **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

537 **a** ++ (10-40% positive cells)

538 **b** +++ (>40% positive cells)

539 **c** - (<5% positive cells).

540 Different groups are classified as described by Screven et al 2014

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559 **Table 5. Gene expression analysis of pAT-MSC and sAT-**
 560 **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

561 **a** – (not expressed)
 562 **b** + (<25% of GAPDH signal)
 563 **c** ++ (25-50% of GAPDH signal)
 564 **d** +++ (>50% of GAPDH signal)

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581 **Table 6. Comparison of the immunophenotypic profile of**
 582 **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
Horseⁱ	/ ^d	- ^a	+++ ^c	- ^a	- ^a	+++ ^c	- ^a
Cat^j	/ ^d	- ^a	+++ ^c	- ^a	/ ^d	+++ ^c	/ ^d

583 **a** - (<5% positive cells)

584 **b** ++ (10-40% positive cells)

585 **c** +++ (>40% positive cells)

586 **d** / (not known)

587 Different groups are classified as described by Screven et al 2014

588 **e** (Dominici et al., 2006)

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

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

591 **h** *our results*

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

593 **j** (Kono et al., 2014)

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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 ^aTakemitsu et al., 2012

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626 **Figure 1** In vitro tri-lineage cell differentiation

627 Photomicrographs of canine perivisceral adipose tissue derived
628 MSC- (pAT-MSC: **b, f, j**) and canine subcutaneous adipose
629 tissue derived MSC (sAT-MSC: **d, g, l**), after 21 days of
630 culture in adipogenic (**b, d**), chondrogenic (**f, h**), osteogenic
631 (**j, l**) induction medium. Parallely a control culture of pAT-MSC
632 (**a, e, i**) and sAT-MSC (**c, g, k**) was grown for 21 days in
633 DMEM. Adipogenic differentiation is evidenced with the
634 presence of intracellular vacuoles colored in red (black arrow)
635 with Oil Red O staining, in pAT-MSC (**b**) and sAT- MSC (**d**),
636 which are absent in control groups (**a, c**) (40X, scale bar
637 200 μ m). Alcian blue staining indicated the presence of
638 aggregates of proteglycans present in treated pAT-MSC (**f**) and
639 sAT-MSC (**h**), and their absence in the control cultures (**e, h**)
640 (10X, scale bar 100 μ m). Osteogenic differentiation was
641 indicated with extracellular calcium aggregates stained with
642 von Kossa coloration in pAT-MSC (**j**) and sAT-MSC (**l**)
643 stimulated with osteogenic medium. Control groups did not
644 contain any calcium aggregates (**i, k**) (10X, scale bar 100 μ m).

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646 **Figure 2** Flow cytometry analysis of the expression of cell
647 surface markers on sAT-MSC and pAT-MSC

648 The expression of cell surface markers is presented in two
649 panels. The upper panel (a) contains data regarding the MSC

650 deriving from subcutaneous adipose tissue and the low panel
651 (b) data about MSC deriving from perivisceral adipose tissue.
652 Live cell populations were gated in forward and side scatter for
653 further analysis. Each histogram contains two peaks. The
654 shaded red peak represents the actual expression of the markers
655 for each cell group, on the other hand the transparent one is the
656 negative control.

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Figure 1
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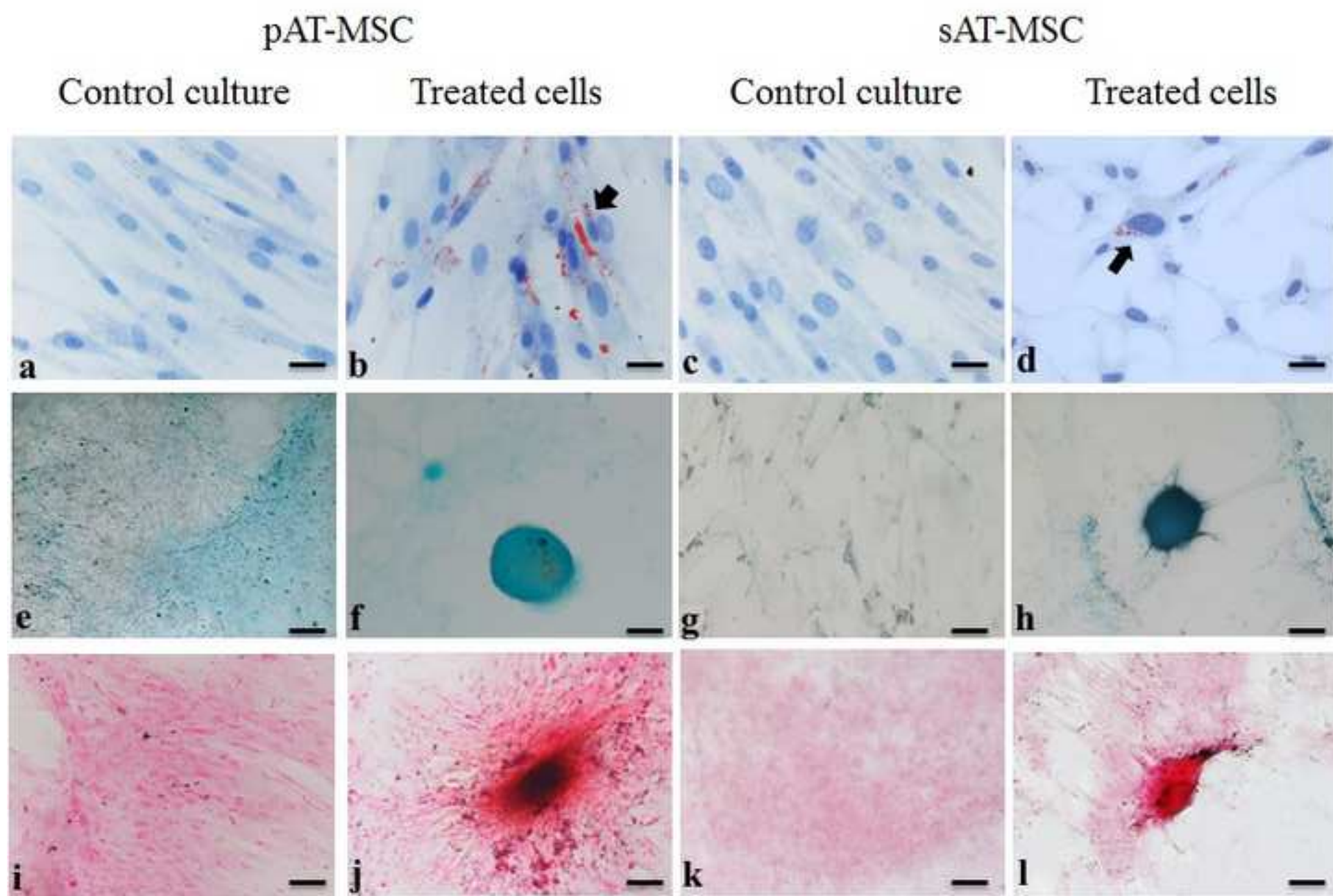
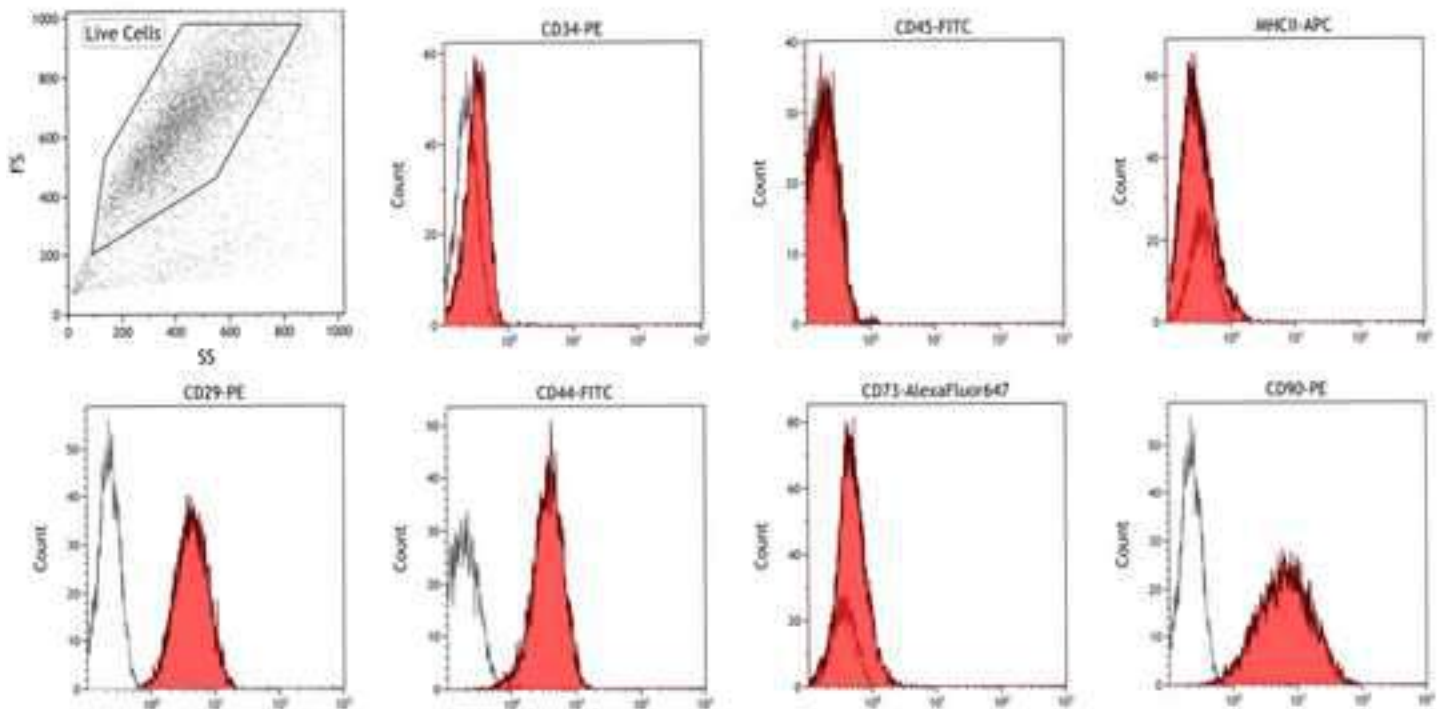


Figure 2

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Expression of cell surface markers on sAT-MSC measured by flow cytometry



Expression of cell surface markers on pAT-MSC measured by flow cytometry

