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

Corresponding Author: Dr. Francesca Ravanetti,

Corresponding Author's Institution: University of Parma

First Author: Ana Ivanovska

Order of Authors: Ana Ivanovska; Stefano Grolli; Paolo Borghetti; Francesca Ravanetti; Virna Conti; Elena De Angelis; Francesca Macchi; Roberto Ramoni; Paolo Martelli; Ferdinando Gazza; Antonio Cacchioli

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

2   stem cells from perivisceral and subcutaneous adipose tissue by

3   a species-specific panel of antibodies

4   **Authors:**

5   Ana Ivanovska[a], Stefano Grolli[a], Paolo Borghetti[a], Francesca

6   Ravanetti[a], Virna Conti[a], Elena De Angelis[a], Francesca

7   Macchi[a], Roberto Ramoni[a], Paolo Martelli[a], Ferdinando Gazza[a]

8   and Antonio Cacchioli[a]

9   [a] Department of Veterinary Science, University of Parma – Via

10   del Taglio 10, 43126, Parma, Italy

11   **Corresponding author:**

12   Dr.Francesca Ravanetti

13   Department of Veterinary Science

14   University of Parma

15   Via del Taglio 10, 43126 Parma

16   Italy

17   00 39 0521 032648

18   francesca.ravanetti@unipr.it

19

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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

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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).~~

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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

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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

7

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 cm^2 (Orange Scientific Tissue Culture

188 Flasks) containing 5 ml of DMEM. The cells were maintained

189 in an incubator at 37°C at 5% CO_2. 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 10^3 cells/cm^2. In each well, 3 ml of DMEM was added and the

200 cells were incubated at 37°C and 5% CO_2. 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 CO_2 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 x 10^3 cells/cm^2. In each well 3 ml of DMEM was added

211 and the cells were incubated at 37°C and 5% CO$_2$. At a

212 confluency of 80% they were treated with with chondrogenic

213 differentiation   mediun   (StemPro   Chondrogenesis

214 Differentiation Kit). The flasks were then put in an incubator

215 with humidified atmosphere of 5% CO$_2$ 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 x 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 μl 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-

11

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 µg/ml streptomycin and 0.25 µg/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 5µl 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 $1.5x10^6$ 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 $MgCl_2$,

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*

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

313

3.1.Cell culture and isolation

314

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

15

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, Rich~~

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

16

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).

494

495

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

22

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. 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

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 of 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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Conflict of interest statement

582   **Acknowledgements**

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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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Table 3. Markers used for the evaluation of cell surface

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: GAGATCACCCTAACGCCT 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

			CCA	
			Rv:	
			CACGGAGGAGGAAGCTG	
			AAG	
Oct-4	XM_53883	Fw:		286bp
	0.1	AAGCCTGCAGAAAGACCT	
		G	
		Rv:	
		GTTCGCTTTCTCTTTCGGG	
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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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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)

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 [a] Takemitsu 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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814 Alamoudi , N. M., El Ashiry, E. A., Farsi, N. M., El Derwi, D.

815   A., Atta, H. M., 2014. Treatment of Oral Ulcers in

816   Dogs Using Adipose Tissue-Derived Mesenchymal

817   Stem Cells. J Clin Pediatr Den. 38(3), 215-222.

818 Barberini, D.J., Freitas, N.P.P., Magnoni, M.S., Maia, L.,

819   Listoni, A.J., Heckler, M.C., Sudano, M.J., Golim, M.A.,

820   da Cruz Landim-Alvarenga, F., Amorim, R.M., 2014.

821   Equine mesenchymal stem cells from bone marrow,

822   adipose tissue and umbilical cord: immunophenotypic

823   characterization and differentiation potential. Stem Cell

824   Res. Ther. 5, 25.

825 Binato, R., de Souza Fernandez, T., Lazzarotto-Silva, C., Du

826   Rocher, B., Mehcalha, A., Pizzatti, L., Bouzas, LF.,

827   Abdelhay, E., 2013. Stability of human mesenchymal stem

828   cells during in vitro culture: considerations for cell

829   therapy. Cell Prolif. 46(1), 10-22.

830 Black, L. L., Gaynor , J., Adams, C., Dhupa, S., Sams, A. E.,

831   Taylor, R., Harman, S., Gingerich, D., Harman, R.,

832   2008. Effect of Intraarticular Injection of Autologous

833   Adipose-Derived Mesenchymal Stem and Regenerative

834   Cells on Clinical Signs of Chronic Osteoarthritis of the

835   Elbow Joint in Dogs. Vet. Ther. 9(3), 192-200.

836 de Mattos Carvalho, A., Alves, A.L.G., Golim, M.A., Moroz,

837   A., Hussni, C.A., de Oliveira, P.G.G., Deffune, E., 2009.

838   Isolation and immunophenotypic characterization of

839   mesenchymal stem cells derived from equine species

840   adipose tissue. Vet. Immunol. Immunopathol. 132, 303–

841   306.

842   Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I.,

843   Marini, F., Krause, D., Deans, R., Keating, A, Prockop,

844   D., Horwitz, E., 2006. Minimal criteria for defining

845   multipotent mesenchymal stromal cells. The International

846   Society for Cellular Therapy position statement.

847   Cytotherapy. 8(4), 315-317.

848   Gharibi, B. and Hughes, F.J., 2012. Effects of Medium

849   Supplements on Proliferation, Differentiation Potential,

850   and In Vitro Expansion of Mesenchymal Stem Cells. Stem

851   Cells Transl Med. 1, 771-782.

852   Guercio, A., Di Bella,S., Casella, S., Di Marco, P., Russo, C.,

853   Piccione G., 2013. Canine Mesenchymal Stem Cells

854   (MSCs): characterization in relation to donor age and

855   adipose tissue-   harvesting site. Cell. Biol. Int. 37(8),

856   789-98.

857   Hall, M. N., Rosenkrantz, W. S., Hong, J. H., Griffin, C. E., &

858   Mendelsohn, C. M., 2010. Evaluation of the Potential

859   Use of Adipose-Derived Mesenchymal Stromal Cells in

860   the Treatment of Canine Atopic Dermatitis: a Pilot

861   Study. Vet.Ther. 11(2), E1-14.

862 Han, S.M., Han, S.H., Coh, Y.R., Jang, G., Chan, R.J., Kang,

863   S.K., Lee, H.W., Youn, H.Y. 2014. Enhanced

864   proliferation and differentiation of Oct-4- and Sox2-

865   overespressing human adipose tissue mesenchymal stem

866   cells. 20(46), 1-9.

867 Hoffman, A.M., Dow, S.W., 2016. Concise review: Stem Cell

868   Trials Using Companion Animal Disease Models. Stem

869   Cells. 34(7), 1709-1729.

870 Kim, Y., Lee, S. H., Kim, W. H., Kweon, O.-K., 2015.

871   Transplantation of Adipose Derived Mesenchymal Stem

872   Cells for Acute Thoracolumbar Disc Disease with no

873   Deep Pain Perception in Dogs. J. Vet. Sci. 17(1), 123-

874   126.

875 Kono, S., Kazama, T., Kano, K., Harada, K., Uechi, M.,

876   Matsumoto, T., 2014. Phenotypic and functional

877   properties of feline dedifferentiated fat cells and adipose-

878   derived stem cells. Vet. J. 199, 88–96.

879 Laschke, M.W., Schank, T.E., Scheuer, C., Kleer, S., Schuler,

880   S., Metzger, W., Eglin, D., Alini, M., Menger, M.D.,

881   2013. Three-dimensional spheroids of adipose-derived

882   mesenchymal stem cells are potent initiators of blood

883   vessel formation in porous polyurethane scaffolds. Acta

884   Biomater. 9, 6876–6884.

885   Lee, S. H., Kim, Y., Rhew, D., Kuk, M., Kim, M., Kweon , O.

886   K., 2015. Effect of the Combination of Mesenchymal

887   Stromal Cells and Chondroitinase ABC on Chronic

888   Spinal Cord Injury. Cytotherapy, 17(10), 1374-1383.

889   Lotfy, A., Salama, M., Zahran, F., Jones, E., Badawy, A., Sobh,

890   M., 2014. Characterization of mesenchymal stem cells

891   derived from rat bone marrow and adipose tissue: a

892   comparative study. Int J Stem Cells, 7, 135–142.

893   Malagola, E., Teunissen, M., van der Laan, L.J., Verstegen,

894   M.M., Schotanus, B.A., van Steenbeek, F.G., Penning,

895   L.C., van Wolferen, M.E., Tryfonidou, M.A., Spee, B,.

896   2016. Characterization and Comparison of Canine

897   Multipotent Stromal Cells Derived from Liver and Bone

898   Marrow. Stem Cells Dev., 25(2), 139-150.

899   Marx, C., Silveira , M. D., Selbach, I., da Silva, A. S., Braga, L.

900   M., Camassola, M., Nardi, N.B. 2014. Acupoint

901   Injection of Autologous Stromal Vascular Fraction and

902   Allogeneic Adipose-Derived Stem Cells to Treat Hip

903   Dysplasia in Dogs. Stem Cells Int. 391274.

904   Marx, C., Silveira, M.D., Beyer Nardi, N., 2015. Adipose-

905   derived stem cells in veterinary medicine: characterization

906   and therapeutic applications. Stem Cells Dev. 24, 803–

907   813.

908   Neupane, M., Chang, C.C., Kiupel, M., Yuzbasiyan-Gurkan,

909   V., 2008. Isolation and characterization of canine adipose-

910   derived mesenchymal stem cells. Tissue Eng Part A.,

911   14(6), 1007-1015.

912   Nery, A.A., Nascimento, I.C., Glaser, T., Bassaneze, V.,

913   Krieger, J.E., Ulrich, H., 2013. Human mesenchymal stem

914   cells: from immunophenotyping by flow cytometry to

915   clinical applications. Cytometry. A 83, 48–61

916   Pascucci, L., Curina, G., Mercati, F., Marini, C., Dall'Aglio, C.,

917   Paternesi, B., Ceccarelli, P., 2011. Flow cytometric

918   characterization of culture expanded multipotent

919   mesenchymal stromal cells (MSCs) from horse adipose

920   tissue: Towards the definition of minimal stemness

921   criteria. Vet. Immunol. Immunopathol. 144, 499–506.

922   Penha, E. M., Meira, C. S., Guimaraes, E. T., Pinheiro

923   Mendoca, M. V., Gravely, F. A., Bahia Pinheiro, C. M.,

924   Bahia Pinheiro, T.M., Barrouin-Melo, S.M., Ribeiro-

925   Dos-Santos, R., Pereira Soares, M.B., 2014 Use of

926   Autologous Mesenchymal Stem Cells Derived from

927   Bone Marrow for the Treatment of Naturally Injured

928   Spinal Cord in Dogs. Stem Cells Int. 437521.

929 Perez-Merino, E. M., Uson-Casaus, J. M., Zaragoza-Bayle, C.,

930   Duque-Carrasco, J., Marinas-Pardo, L., Hermida-Prieto,

931   M., Barrera-Chacon, R., Gualtieri, M., 2015. Safety and

932   Efficacy of Allogeneic Adipose Tissue-Derived

933   Mesenchymal Stem Cells for Treatment of Dogs with

934   Inflammatory Bowel Disease: Clinical and Laboratory

935   Outcomes. Vet J. 206, 385-390.

936 Pogue , B., Estrada, A. H., Sosa-Samper, I., Maisenbacher , H.

937   W., Lamb, K. E., Mincey, B. D., Erger, K.E., Conlon,

938   T.J., 2013. Stem-Cell Therapy for Dilated

939   Cardiomyopathy: a Pilot Study Evaluating Retrograde

940   Coronary Venous Delivery. J. Small Anim. Pract.

941   54(7), 361-366.

942 Reich, C.M., Raabe, O., Wenisch, S., Bridger, P.S., Kramer,

943   M., Arnhold,S. 2012. Isolation, culture and

944   chondrogenic differentiation of canine adipose tissue-

945   and bone marrow-derived mesenchymal stem cells-a

946   comparative study.Vet Res Commun. 36(2), 139-148.

947 Rozemuller, H., Prins, H., Naaijkens, B., Staal, J., Bühring, H.,

948   Martens,   A.C.,   2010.   Prospective   Isolation   of

949   Mesenchymal   Stem   Cells   from   Multiple   Mammalian

950   Species   Using   Cross-Reacting   Anti-Human   Monoclonal

951   Antibodies. Stem Cells Dev. 19(12), 1911-1921.

952 Russell, K.A., Chow, N.H., Dukoff, D., Gibson, T.W.,

953   LaMarre, J., Betts, D.H., Koch, T.G. 2016.

954   Characterization and immunomodulatory effects of canine

955   adipose tissue and bone marrow-derived mesenchymal

956   stromanl cells. PLoS One, 11(12), 1-18.

957 Screven, R., Kenyon, E., Myers, M.J., Yancy, H.F., Skasko,

958   M., Boxer, L., Bigley III, E.C., Borjesson, D.L., Zhu,

959   M., 2014. Immunophenotype and gene expression

960   profiles of mesenchymal stem cells derived from canine

961   adipose tissue and bone marrow. Vet. Immunol.

962   Immunopathol. 161, 21–31.

963 Taha, M.F., Hedayati, V., 2010. Isolation, identification and

964   multipotential differentiation of mouse adipose tissue-

965   derived stem cells. Tissue Cell 42, 211–216.

966 Takemitsu H, Zhao D, Yamamoto I, Harada Y, Michishita M,

967   A.T., 2012. Comparison of bone marrow and adipose

968   tissue-derived canine mesenchymal stem cells. BMC Vet

969   Res. 8, 150.

970 Tapp, H., Deepe, R., Ingram, J.A., Kuremsky, M., Hanley,

971   E.N., Gruber, H.E., 2008. Adipose-derived mesenchymal

972   stem cells from the sand rat: transforming growth factor

973   beta and 3D co-culture with human disc cells stimulate

974   proteoglycan and collagen type I rich extracellular matrix.

975   Arthritis Res. Ther. 10, R89.

976   Villatoro, A., Fernández, V., 2014. Use of Adipose-Derived

977   Mesenchymal Stem Cells in Keratoconjunctivitis Sicca in

978   a Canine Model. Biomed Res. Int. 2015, 527926.

979   Volk, S.W., Theoret, C., 2013. Translating stem cell therapies:

980   the role of companion animals in regenerative medicine.

981   Wound Repair Regen. 21, 382–394.

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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

4   **Authors:**

5   Ana Ivanovska[a], Stefano Grolli[a], Paolo Borghetti[a], Francesca

6   Ravanetti[a], Virna Conti[a], Elena De Angelis[a], Francesca

7   Macchi[a], Roberto Ramoni[a], Paolo Martelli[a], Ferdinando Gazza[a]

8   and Antonio Cacchioli[a]

9   [a] Department of Veterinary Science, University of Parma – Via

10   del Taglio 10, 43126, Parma, Italy

11   **Corresponding author:**

12   Dr.Francesca Ravanetti

13   Department of Veterinary Science

14   University of Parma

15   Via del Taglio 10, 43126 Parma

16   Italy

17   00 39 0521 032648

18   francesca.ravanetti@unipr.it

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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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1. Introduction

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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.

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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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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

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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 cm^2 (Orange Scientific Tissue Culture

171 Flasks) containing 5 ml of DMEM. The cells were maintained

172 in an incubator at 37°C at 5% CO_2. 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 10^3 cells/cm^2. In each well, 3 ml of DMEM was added and the

183 cells were incubated at 37°C and 5% CO_2. At a confluency of

184 80% they were treated with adipogenic differentiation media

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185   (StemPro Adipogenesis Differentiation Kit). The flasks were

186   then put in an incubator with humidified atmosphere of 5%

187   CO_2 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 x 10^3 cells/cm^2. In each well 3 ml of DMEM was added

194   and the cells were incubated at 37°C and 5% CO_2. At a

195   confluency of 80% they were treated with with chondrogenic

196   differentiation   mediun   (StemPro   Chondrogenesis

197   Differentiation Kit). The flasks were then put in an incubator

198   with humidified atmosphere of 5% CO_2 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 x 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

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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 µl 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 SytoxAAdvanced

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 µg/ml streptomycin and 0.25 µg/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 5µl 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 $MgCl_2$,

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 periviscelar-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**

464 **Acknowledgements**

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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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_0011 46034.1	Fw: GGTCCTTACCATCACCTGGC Rv: CCTAAGGCCATCCATCGTCC	335bp
CD 29	XM_0056 16949.1	Fw: AGGATGTTGACGACTGCTGG Rv: ACCTTTGCATTCAGTGTTGTGC	356bp
CD 31	XM_8483 26	Fw: GCCCGAAGTTCACTCTCAAG Rv:CACTCCTTTGACCCACACCT	410bp
CD 34	NM_0010 03341.1	Fw: GAGATCACCCTAACGCCTGG Rv: GGCTCCTTCTCACACAGGAC	383bp
CD 44	NM_0011 97022.1	Fw: CCCATTACCAAAGACCACGA Rv: TTCTCGAGGTTCCGTGTCTC	408bp
CD 45	XM_0056 22282.1	Fw: TGTTTCCAGTTCTGTTTCCCCA Rv: TCAGGTACAAAGCCTTCCCA	432bp
CD 73	XM_5322 21.4	Fw: GATGGGAAAGGCAAGAGGCT Rv: TTCCTGGCATCTTGCTACGG	317bp
CD 90	NM_0012 87129.1	Fw: AAGCCAGGATTGGGGATGTG Rv: TGTGGCAGAGAAAGCTCCTG	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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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.

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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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)

Table 7 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

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   200µm). 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 100µm). 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 ostegenic medium. Control groups did not

644   contain any calcium aggregates (**i, k**) (10X, scale bar 100um).

645

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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675   Alamoudi , N. M., El Ashiry, E. A., Farsi, N. M., El Derwi, D.

676   A., Atta, H. M., 2014. Treatment of Oral Ulcers in

677   Dogs Using Adipose Tissue-Derived Mesenchymal

678   Stem Cells. J Clin Pediatr Den. 38(3), 215-22.

679   Barberini, D.J., Freitas, N.P.P., Magnoni, M.S., Maia, L.,

680   Listoni, A.J., Heckler, M.C., Sudano, M.J., Golim, M.A.,

681   da Cruz Landim-Alvarenga, F., Amorim, R.M., 2014.

682   Equine mesenchymal stem cells from bone marrow,

683   adipose tissue and umbilical cord: immunophenotypic

684   characterization and differentiation potential. Stem Cell

685   Res. Ther. 5, 25.

686   Black, L. L., Gaynor , J., Adams, C., Dhupa, S., Sams, A. E.,

687   Taylor, R., Harman, S., Gingerich, D., Harman, R.,

688   2008. Effect of Intraarticular Injection of Autologous

689   Adipose-Derived Mesenchymal Stem and Regenerative

690   Cells on Clinical Signs of Chronic Osteoarthritis of the

691   Elbow Joint in Dogs. Vet. Ther. 9(3), 192-200.

692   de Mattos Carvalho, A., Alves, A.L.G., Golim, M.A., Moroz,

693   A., Hussni, C.A., de Oliveira, P.G.G., Deffune, E., 2009.

694   Isolation and immunophenotypic characterization of

695   mesenchymal stem cells derived from equine species

696   adipose tissue. Vet. Immunol. Immunopathol. 132, 303–

697   306.

698   Del Bue, M., Riccò, S., Ramoni, R., Conti, V., Grolli, S., 2008.

699   Equine Adipose-Tissue Derived Mesenchymal Stem

700   Cells and Platelet Concentrates: Their Association In

701   Vitro and In Vivo. Vet. Ress. Commun. 32(Suppl 1),

702   S51-S55.

703   Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I.,

704   Marini, F., Krause, D., Deans, R., Keating, a, Prockop,

705   D., Horwitz, E., 2006. Minimal criteria for defining

706   multipotent mesenchymal stromal cells. The International

707   Society for Cellular Therapy position statement.

708   Cytotherapy 8, 315–7.

709   Govoni, K. E., 2015. Horse Species Symposium: Use of

710   Mesenchymal Stem Cells in Fracture Repair in Horses.

711   J. Anim. Sci. 93(3), 871-878.

712   Guercio, A., Di Bella,S., Casella, S., Di Marco, P., Russo, C.,

713   Piccione G., 2013. Canine Mesenchymal Stem Cells

714   (MSCs): characterization in relation to donor age and

715   adipose tissue- harvesting site. Cell. Biol. Int. 37(8):

716   789-98.

717   Hall, M. N., Rosenkrantz, W. S., Hong, J. H., Griffin, C. E., &

718   Mendelsohn, C. M., 2010. Evaluation of the Potential

719   Use of Adipose-Derived Mesenchymal Stromal Cells in

720   the Treatment of Canine Atopic Dermatitis: a Pilot

721   Study. Vet.Ther. 11(2), E1-14.

722 Kim, Y., Lee, S. H., Kim, W. H., Kweon, O.-K., 2015.

723   Transplantation of Adipose Derived Mesenchymal Stem

724   Cells for Acute Thoracolumbar Disc Disease with no

725   Deep Pain Perception in Dogs. J. Vet. Sci. 17(1), 123-

726   126.

727 Kono, S., Kazama, T., Kano, K., Harada, K., Uechi, M.,

728   Matsumoto, T., 2014. Phenotypic and functional

729   properties of feline dedifferentiated fat cells and adipose-

730   derived stem cells. Vet. J. 199, 88–96.

731 Laschke, M.W., Schank, T.E., Scheuer, C., Kleer, S., Schuler,

732   S., Metzger, W., Eglin, D., Alini, M., Menger, M.D.,

733   2013. Three-dimensional spheroids of adipose-derived

734   mesenchymal stem cells are potent initiators of blood

735   vessel formation in porous polyurethane scaffolds. Acta

736   Biomater. 9, 6876–6884.

737 Lee, S. H., Kim, Y., Rhew, D., Kuk, M., Kim, M., Kweon , O.

738   K., 2015. Effect of the Combination of Mesenchymal

739   Stromal Cells and Chondroitinase ABC on Chronic

740   Spinal Cord Injury. Cytotherapy, 17(10), 1374-83.

741 Lotfy, A., Salama, M., Zahran, F., Jones, E., Badawy, A., Sobh,

742   M., 2014. Characterization of mesenchymal stem cells

743 derived from rat bone marrow and adipose tissue: a

744 comparative study. Int J Stem Cells 7, 135–142.

745 Martinello, T., Bronzini, I., Maccatrozzo, L., Mollo, A.,

746 Sampaolesi, M., Mascarello, F., Decaminada, M., Patruno,

747 M., 2011. Canine adipose-derived-mesenchymal stem

748 cells do not lose stem features after a long-term

749 cryopreservation. Res. Vet. Sci. 91, 18–24.

750 Marx, C., Silveira , M. D., Selbach, I., da Silva, A. S., Braga, L.

751 M., Camassola, M., Nardi, N.B. 2014. Acupoint

752 Injection of Autologous Stromal Vascular Fraction and

753 Allogeneic Adipose-Derived Stem Cells to Treat Hip

754 Dysplasia in Dogs. Stem Cells Int. 391274.

755 Marx, C., Silveira, M.D., Beyer Nardi, N., 2015. Adipose-

756 derived stem cells in veterinary medicine: characterization

757 and therapeutic applications. Stem Cells Dev. 24, 803–13.

758 Nery, A. a, Nascimento, I.C., Glaser, T., Bassaneze, V.,

759 Krieger, J.E., Ulrich, H., 2013. Human mesenchymal stem

760 cells: from immunophenotyping by flow cytometry to

761 clinical applications. Cytometry. A 83, 48–61.

762 Paebst, F., Piehler, D., Brehm, W., Heller, S., Schroeck, C.,

763 Tarnok, A., Burk, J., 2014. Comparative

764 Immunophenotyping of Equine Multipotent

765 Mesenchymal Stromal Cells: An Approach Toward a

766   Standardized Definition. Cytometry Part A(85A), 678-

767   687.

768   Pascucci, L., Curina, G., Mercati, F., Marini, C., Dall'Aglio, C.,

769   Paternesi, B., Ceccarelli, P., 2011. Flow cytometric

770   characterization of culture expanded multipotent

771   mesenchymal stromal cells (MSCs) from horse adipose

772   tissue: Towards the definition of minimal stemness

773   criteria. Vet. Immunol. Immunopathol. 144, 499–506.

774   Penha, E. M., Meira, C. S., Guimaraes, E. T., Pinheiro

775   Mendoca, M. V., Gravely, F. A., Bahia Pinheiro, C. M.,

776   Bahia Pinheiro, T.M., Barrouin-Melo, S.M., Ribeiro-

777   Dos-Santos, R., Pereira Soares, M.B., 2014 Use of

778   Autologous Mesenchymal Stem Cells Derived from

779   Bone Marrow for the Treatment of Naturally Injured

780   Spinal Cord in Dogs. Stem Cells Int. 437521.

781   Perez-Merino, E. M., Uson-Casaus, J. M., Zaragoza-Bayle, C.,

782   Duque-Carrasco, J., Marinas-Pardo, L., Hermida-Prieto,

783   M., Barrera-Chacon, R., Gualtieri, M., 2015. Safety and

784   Efficacy of Allogeneic Adipose Tissue-Derived

785   Mesenchymal Stem Cells for Treatment of Dogs with

786   Inflammatory Bowel Disease: Clinical and Laboratory

787   Outcomes. Vet J. 206, 385-390.

788   Pogue , B., Estrada, A. H., Sosa-Samper, I., Maisenbacher , H.

789   W., Lamb, K. E., Mincey, B. D., Erger, K.E., Conlon,

790   T.J., 2013. Stem-Cell Therapy for Dilated

791   Cardiomyopathy: a Pilot Study Evaluating Retrograde

792   Coronary Venous Delivery. J. Small Anim. Pract.

793   54(7), 361-6.

794 Quimby, J. M., Webb, T. L., Randall, E., Marolf, A., Valdes-

795   Martinez, A., Dow, S. W., 2016. Assessment of

796   Intravenous Adipose-Derived Allogeneic Mesenchymal

797   Stem Cells for the Treatment of Feline Chronic Kidney

798   Disease: a Randomized , Placebo-Controlled Clinical

799   Trial in Eight Cats. J. Feline Med. Surg. 18(2), 165-171.

800 Rich, F. R., 2014. Single-Center Study of 83 Horses with

801   Suspensory Injuries Treated with Adipose-Derived

802   Stem and Regenerative Cells. Stem Cell Discovery, 4,

803   44-53.

804 Screven, R., Kenyon, E., Myers, M.J., Yancy, H.F., Skasko,

805   M., Boxer, L., Bigley III, E.C., Borjesson, D.L., Zhu, M.,

806   2014. Immunophenotype and gene expression profiles of

807   mesenchymal stem cells derived from canine adipose

808   tissue and bone marrow. Vet. Immunol. Immunopathol.

809   161, 21–31.

810 Taha, M.F., Hedayati, V., 2010. Isolation, identification and

811   multipotential differentiation of mouse adipose tissue-

812   derived stem cells. Tissue Cell 42, 211–216.

813   Takemitsu H, Zhao D, Yamamoto I, Harada Y, Michishita M,

814   A.T., 2012. Comparison of bone marrow and adipose

815   tissue-derived canine mesenchymal stem cells. BMC Vet

816   Res 8.

817   Tapp, H., Deepe, R., Ingram, J.A., Kuremsky, M., Hanley,

818   E.N., Gruber, H.E., 2008. Adipose-derived mesenchymal

819   stem cells from the sand rat: transforming growth factor

820   beta and 3D co-culture with human disc cells stimulate

821   proteoglycan and collagen type I rich extracellular matrix.

822   Arthritis Res. Ther. 10, R89.

823   Trzil, J. E., Masseau, I., Webb, T. L., Chang , C. H., Dodam, J.

824   R., Liu, H., Quimby, J.M., Dow, S.W., Reinero, C.R.,

825   2015. Intravenous Adipose-Derived Mesenchymal Stem

826   Cell Therapy for the Treatment of Feline Asthma : A

827   Pilot Study. J. Feline Med. Surg.*17*.

828   Villatoro, A., Fernández, V., 2014. Use of Adipose-Derived

829   Mesenchymal Stem Cells in Keratoconjunctivitis Sicca in

830   a Canine Model. Biomed Res. Int. 2015, 10.

831   Volk, S.W., Theoret, C., 2013. Translating stem cell therapies:

832   the role of companion animals in regenerative medicine.

833   Wound Repair Regen. 21, 382–94.

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Figure 1
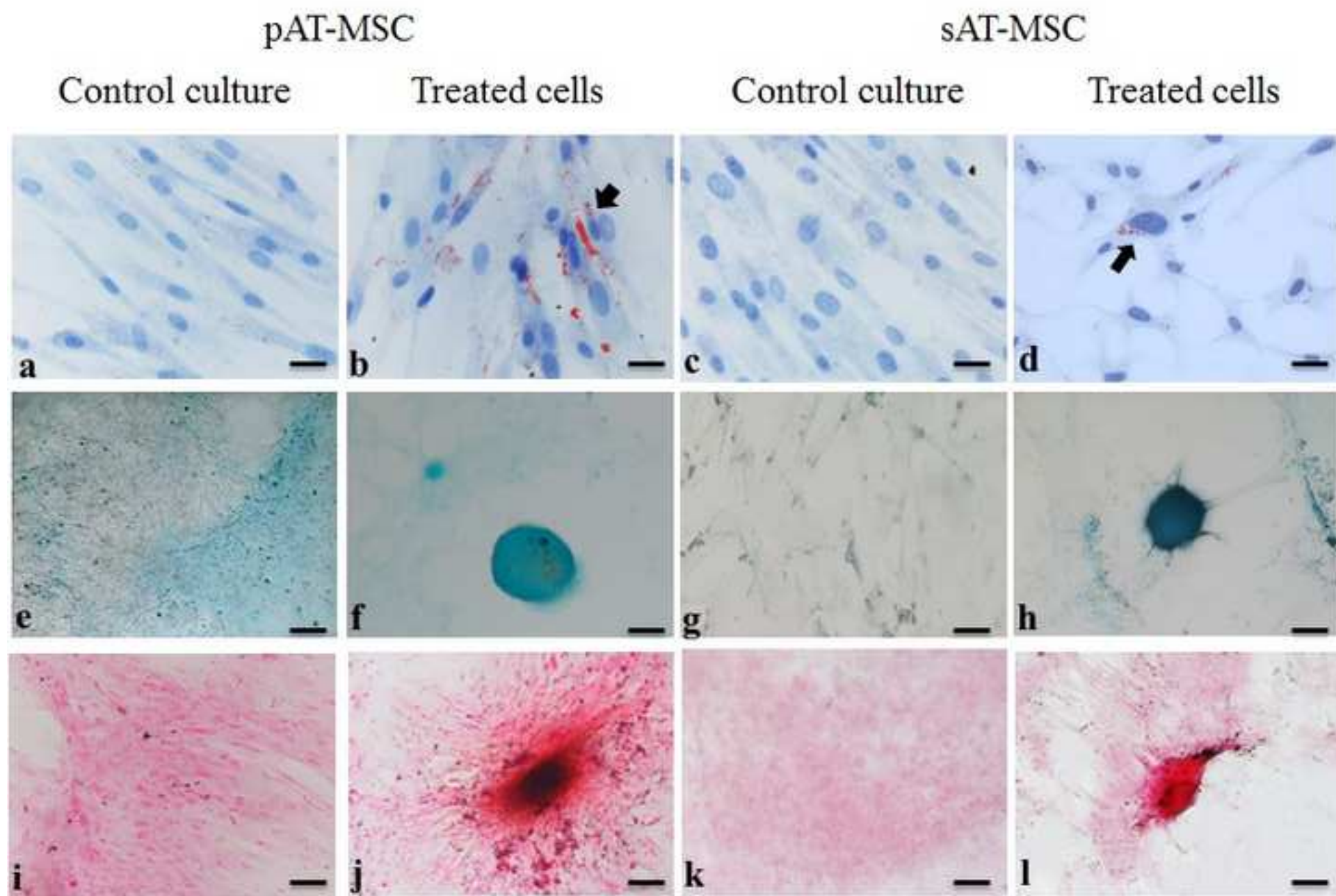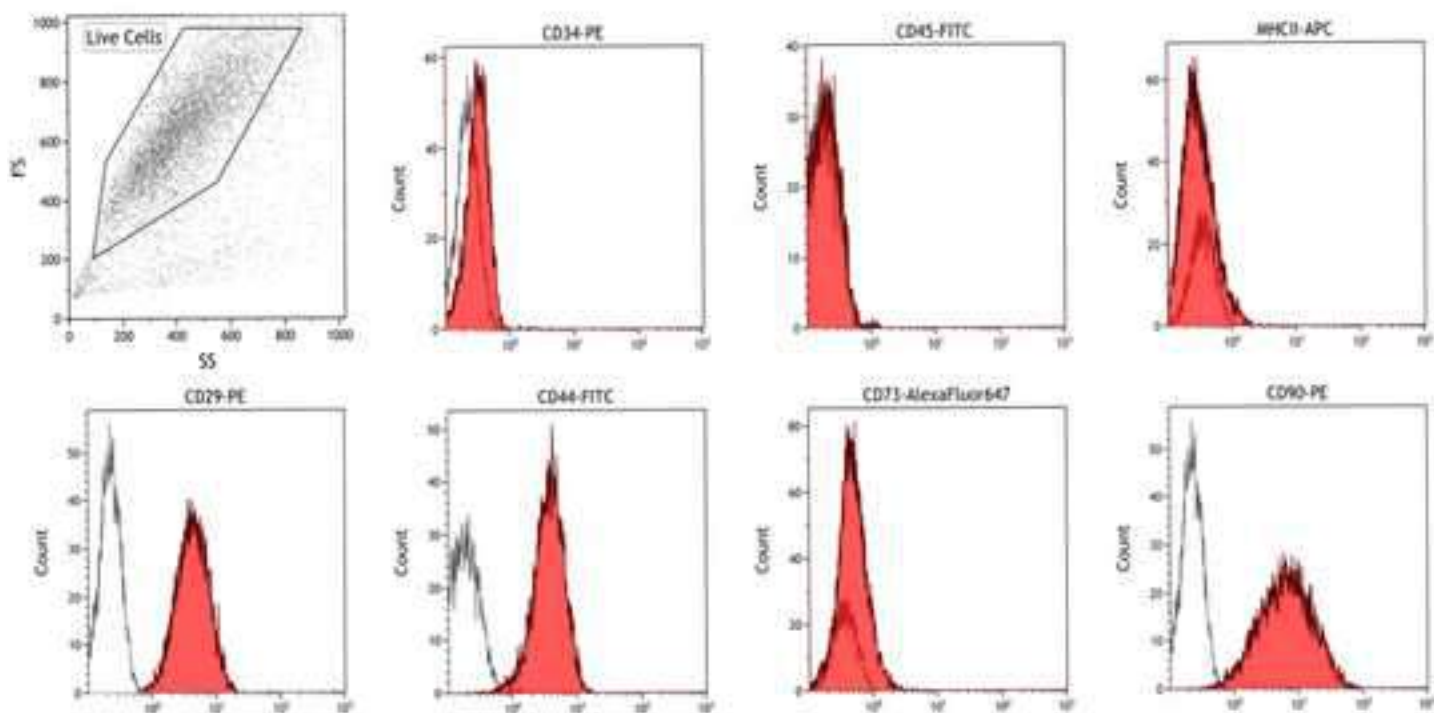**Click here to download high resolution image**

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