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Gene expression markers in horse articular chondrocytes: Chondrogenic differentiation IN VITRO depends on the proliferative potential and ageing. Implication for tissue engineering of cartilage

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GENE EXPRESSION MARKERS IN HORSE ARTICULAR CHONDROCYTES:
CHONDROGENIC DIFFERENTIATION *IN VITRO* DEPENDS ON THE PROLIFERATIVE
POTENTIAL AND AGEING. IMPLICATION FOR TISSUE ENGINEERING OF CARTILAGE.

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22 **Abstract**

23 Chondrocyte dedifferentiation is a key limitation in therapies based on autologous chondrocyte
24 implantation for cartilage repair. Articular chondrocytes, obtained from (metacarpophalangeal and
25 metatarsophalangeal) joints of different aged horses, were cultured in monolayer for several passages
26 (P0 to P8). Cumulative Populations Doublings Levels (PDL) and gene expression of relevant
27 chondrocyte phenotypic markers were analysed during culturing. Overall data confirmed that, during
28 proliferation *in vitro*, horse chondrocytes undergo marked morphological and phenotypic alterations
29 of their differentiation status. Particularly, the dedifferentiation started early in culture (P0-P1) and
30 was very marked at P3 subculture (PDL 4-6): proliferative phase after P3 could be critical for
31 maintenance/loss of differentiation potential. In elderly animals, chondrocytes showed aspects of
32 dedifferentiation shortly after their isolation, associated with reduced proliferative capacity.
33 Regarding the gene expression of major cartilage markers (Col2, Aggrecan, SOX9) there was a very
34 early reduction (P1) in proliferating chondrocytes independent of age. The chondrocytes from adult
35 donors showed a more stable expression (up to P3) of some (Col6, Fibromodulin, SOX6, TGβ1)
36 markers of mature cartilage; these markers could be tested as parameter to determine the
37 dedifferentiation level. This study can provide parameters to identify up to which "culture step"
38 chondrocytes for implantation with a conserved phenotypic potential can be obtained, and to test the
39 efficiency of biomaterial scaffold or chondroinductive media/signals to maintain/recover the
40 chondrocyte phenotype. Moreover, the determination of levels and time related expression of these
41 markers can be useful during the chondroinduction of mesenchymal stem cells.

42

43

44 **Keywords:** Chondrocytes, Proliferative Potential, Gene Expression, Foals, Adult, Elderly horses.

45 **1. INTRODUCTION**

46 Osteoarthritis (OA) in animals and humans causes progressive damage of articular cartilage with
47 progressive tissue degeneration/destruction. In OA tissue there is increased production of
48 metalloproteases (MMP) with loss of proteoglycans and collagen cleavage linked to signals leading
49 to an altered differentiation and death of chondrocytes, resulting in the inability to produce a proper
50 newly synthesized extracellular matrix (Garvican, Vaughan-Thomas, Redmond, & Clegg, 2008;
51 Goldring, 2012; Troeberg & Nagase, 2012).

52 Indeed, once damaged, the articular cartilage is unable to heal spontaneously and the repair process
53 leads to the formation of a fibro-cartilaginous tissue which does not have the native mechanical
54 properties of hyaline cartilage (Goldring & Goldring, 2007; McIlwraith, Frisbie, & Kawcak, 2012).

55 Furthermore, the increased age of animals is an important risk factor of OA because the senescence
56 of chondrocytes results in the development of secretory phenotype and ageing changes in the matrix
57 (Rahmati, Nalesso, Mobasher, & Mozafari, 2017; Shane Anderson & Loeser, 2010).

58 Attempts to solve this issue have stimulated the development of studies focused on innovative
59 pharmacological therapies (chondro-inductive drugs) or tissue engineering methods in order to
60 modulate the chondrocytes differentiation and their synthetic activity (Goldring, 2012; Pulsatelli,
61 Addimanda, Brusi, Pavloska, & Meliconi, 2012; Vinatier & Guicheux, 2016).

62 As an option for the repair of the injured cartilage, currently, MACI (“Matrix-associated Chondrocyte
63 Implantation”), provides a helpful approach in cartilage repair treatment using synthetic and natural
64 polymer materials, both in human (Brittberg, 2010; Demoor et al., 2014; Kon, Filardo, Di Matteo,
65 Perdisa, & Marcacci, 2013; Niemeyer et al., 2013) and veterinary medicine, specifically equine,
66 (Barnewitz et al., 2006; J. pil Seo et al., 2015), with some promising but variable results, and further
67 studies on methods and techniques are needed to improve the quality of repaired tissue (Frisbie,
68 McCarthy, Archer, Barrett, & McIlwraith, 2015; Frisbie & Stewart, 2011; Kyla F. Ortved, Begum,
69 Mohammed, & Nixon, 2015; Vindas Bolaños et al., 2017).

70 Usually in MACI, the autologous chondrocytes are isolated from a very small quantity of cartilage
71 (biopsy) and need to be expanded *in vitro* in monolayer culture to obtain enough cells for seeding in

72 a three-dimensional biodegradable scaffold; the chondrocyte-loaded scaffold is then reimplanted into
73 the defect to induce the formation of repair tissue. During monolayer expansion, articular
74 chondrocytes rapidly modify their phenotype, as they undergo a progressive dedifferentiation and
75 assume fibroblast-like cell morphology, characterised with an alteration of the synthesis of typical
76 ECM components. During extended passages, dedifferentiated chondrocytes can lose their capacity
77 to form native matrix cartilage. Thus, apart from the intrinsic chondroinductive capacity of the
78 biomaterial, the control of chondrogenic potential during the expansion phase must be considered as
79 a major condition to increase the chances of successful cartilage regeneration *in vivo*.

80 Although different strategies have been developed to redifferentiate expanded chondrocytes *in vitro*
81 and several of them were able to induce this process at least partially, they did not produce enough
82 matrix molecules to form a hyaline cartilage mature with mechanic function, this process may remain
83 incomplete (Barlič, Drobnič, Maličev, & Kregar-Velikonja, 2008; Jakob et al., 2001; G. Liu et al.,
84 2007; Schnabel et al., 2002; Taylor et al., 2014) and for these reasons further investigations are
85 required.

86 Therefore, it appears critical to assess changes able to influence negatively, at various *in vitro* and *in*
87 *vivo* levels, the success and predictability of the reparative treatment in humans and also in animals
88 (Behery, Harris, Karnes, Siston, & Flanigan, 2013; Nürnberger et al., 2013; Steinert et al., 2007).

89 The cell quality is also related to the age of the patient, since the chondrocytes' senescent reduces
90 until they almost lose their proliferative capacity, and their capacity to maintain/regain differentiation,
91 *in vitro* and also *in vivo*, independently from the characteristics of the biocompatible construct
92 (Bernhard & Vunjak-Novakovic, 2016; Phull, Eo, Abbas, Ahmed, & Kim, 2016).

93 Several studies have suggested that age is an important factor which limits cartilage repair following
94 injury and increases the risk of OA. The actual interest is to identify the entity and mechanism of
95 senescence for assessing novel strategies for modulation of cellular senescence and consequently
96 may help to improve cartilage regeneration in an ageing population. (Toh et al., 2016).

97 Moreover, cell senescence during long-term culture of chondrocytes or tissue-derived mesenchymal
98 stem cells (MSCs), is also a major problem *in vitro* in cellular transplantation for cartilage repair (J.
99 Li & Pei, 2012).

100 Articular cartilage lesions due to injuries which are common in performance/athlete horses and lead
101 to OA, but current cartilage therapy and regenerative medicine are no definitive treatment (Cokelaere,
102 Malda, & van Weeren, 2016; Johnson & Frisbie, 2016; K. F. Orved & Nixon, 2016). Moreover the
103 horse is accepted as an important animal model for joint disease in humans (Wayne McIlwraith,
104 Fortier, Frisbie, & Nixon, 2011) meaning there is a need to develop novel tissue engineering
105 techniques for cartilage healing and restoration functions in horses as well as in humans.

106 Since the mechanisms underlying the dedifferentiation process in articular chondrocytes were poorly
107 studied in horses, we evaluated an extended pattern of gene expression related to cartilage matrix
108 components, aiming to identify markers that may distinguish articular differentiated chondrocytes
109 from dedifferentiated chondrocytes, and to finally characterise chondrocyte dedifferentiation.

110 Aggrecan (ACAN) and Collagen Type II (COL2A1) are the major matrix molecules of mature
111 articular cartilage (Schulz & Bader, 2007) while Collagen I is the largest fibrillar collagen produced
112 by osteoblasts and its expression was observed in joint disorders characterised by an extracellular
113 matrix disorganisation and by the assumption of a typical fibroblast-like phenotype (Semevolos,
114 Nixon, & Brower-Toland, 2001). Other small matrix molecules such as Collagen Type VI (Coll6),
115 Biglycan (BGN), Fibromodulin (FMOD) and Cartilage Oligomeric Matrix Protein (COMP) are
116 mainly involved in the interaction with other macromolecules of mature matrix to maintain the
117 integrity of articular cartilage (Wilda et al. 2000). SOX6/SOX9 and RUNX2 encodes transcription
118 factors, SOX6/SOX9 are essential for early chondrocyte differentiation (Li, Leo, Beck, Balian, &
119 Anderson, 2004) and RUNX 2 for expression of genes is mainly relevant for the development of
120 hypertrophic and terminal chondrocytes (C. F. Liu, Samsa, Zhou, & Lefebvre, 2017). Moreover
121 TGF- β 1 is an anabolic factor for articular chondrocytes that acts through the ALK5/SMAD2 pathway,
122 stimulating chondrocytes to release extracellular matrix molecules (Cheng, Maddox, Wong,

123 Rahnama, & Kuo, 2012) while SMAD1 induces the chondrocytes' progression to hypertrophic
124 condition.

125 Specifically, the aim of the present work was to study the chondrocytes derived from the fetlock
126 equine joint because it is the most frequent site of traumatic injuries, fragmentation, fracture, and
127 necrosis, which can lead to osteoarthritis (Easton & Kawcak, 2007) and to evaluate how *in vitro*
128 proliferation can influence the loss of differentiation of equine articular chondrocytes in relation to
129 the proliferative stage and to the animal's age, by analysing the differentiation through chondrogenic
130 markers related to gene expression.

131

132 **2. MATERIALS and METHODS**

133 **2.1 Animals**

134 The articular cartilage was obtained from healthy metacarpophalangeal and metatarsophalangeal
135 joints of slaughtered horses farmed for human consumption. Animals were classified based on
136 different ages and were randomly allocated to the following groups without any distinction of gender,
137 race or type of use.

138 Group I: Foals aged up to 2 years (N. 15);

139 Group II: Adult horses from 5 to 8 years (N. 15);

140 Group III: Elderly horses over 12 years old (N. 15).

141 Before cartilage harvesting, joints were carefully examined. Joints with macroscopic lesions related
142 to overt OA (focal and extended cartilage erosive lesions, linear wearing lines, osteophytes, capsular
143 fibrosis) or with evidence of synovitis were excluded from the study.

144

145 **2.2 Isolation of chondrocytes from articular cartilage tissue**

146 The chondrocytes were isolated from the articular cartilage and cultured following a previously
147 described protocol (De Angelis et al., 2017). Briefly, the cartilage was finely diced in sterile

148 conditions and then washed several times in phosphate buffered saline (PBS). After pre-incubation
149 in 0.1% pronase (Sigma) solution for 1h at 37° C, the tissue was treated with 0.2% collagenase type
150 IA (Sigma) in D-MEM for 2h at 37°C. The digested tissue was filtered through 100 µm and 20 µm
151 nylon filters and the cellular suspension was centrifuged at 1500 rpm for 10 min. The supernatant
152 was discarded and the pellet was washed several times with D-MEM (4.5gl glucose; 25mM Hepes)
153 containing 10% foetal calf serum (FCS), 100U/ml penicillin and 0.1mg/ml streptomycin. The number
154 of chondrocytes (CHs) was determined using a haemocytometer and the cell viability (>95%) was
155 assessed by Trypan Blue (0.1%) exclusion.

156

157 **2.3 Primary cultures, subculturing and Cumulative Population Doubling Level (CPDL)** 158 **determination**

159 Freshly isolated chondrocytes (P0: Passage 0) were plated (2×10^4 cells/cm²) in triplicate in a Petri
160 dish (57cm²) (Sarstedt, Nümbrecht, Germany) cultured in D-MEM with 4,5 g/l glucose (SIGMA
161 Aldrich) with 10% FCS at 37°C and 5%CO₂. The medium was changed every three days. The cultures
162 (P1: Passage 1) were observed by phase contrast microscopy at 1, 5 and 10 days of culture. After
163 reaching monolayer, chondrocytes were detached with 0,25% trypsin – 0,02% EDTA (SIGMA
164 Aldrich) and counted using a Bürker hemocytometer; then 2×10^4 cells were reseeded in a secondary
165 subculture (P2: Passage 2); the other subcultures (P3, P4, P5 ecc.) were set up until the cells stopped
166 proliferating.

167 To evaluate the cumulated doubling level, the population doubling for each passage was calculated
168 and then added to the population doubling levels of the previous passages. The estimated growth
169 efficiency and proliferation potential of horse articular chondrocytes were determined by CPDL
170 analysis using the formula $[\log_{10}(N_H) - \log_{10}(N_I)] / \log_{10}(2) = X$] where N_H = inoculum number, N_I
171 = cell harvest number, and X = population doublings (PD). The population doubling increase that
172 was calculated was then added to the previous population doubling level (PDL), to yield the
173 cumulative population doubling level (Cristofalo, Allen, Pignolo, Martin, & Beck, 1998).

174

175 **2.4 Gene Expression of Differentiation Markers**

176 ***2.4.1 Total RNA Extraction and cDNA Synthesis.***

177 Total RNA of each sample was extracted using TRIreagent (Ambion, Inc., Austin, TX), according to
178 the manufacturer's instructions, from cells seeded on plastic. Purity and concentration were assessed
179 by UV spectrophotometry at 260/280 and 260 nm respectively (GeneQuant Pro, Amersham
180 Pharmacia Biotech-GE Healthcare Life Sciences, Little Chalfont, UK). RNA integrity and quality
181 were assessed by using the Agilent Bioanalyzer 2100 and the RNA 6000 Labchip kit (Agilent
182 Technologies, Santa Clara, CA, USA).

183 ***2.4.2 Reverse-transcription (RT)***

184 All RNA samples were DNase-treated (Sigma) prior to cDNA synthesis. Total RNA (1 µg/20 µL)
185 was reverse-transcribed using a High-capacity cDNA Reverse Transcription kit (Applied
186 Biosystems, Foster City, CA). The RT was performed according to the manufacturer's instructions,
187 under the following thermal conditions: 10 mins at 25 °C, 120 mins at 37 °C followed by 5 mins at
188 85°C. All cDNA samples were stored at -20 °C until PCR was performed. The cDNA obtained was
189 used as a template for the subsequent polymerase chain reaction.

190 ***2.4.3 Quantification of mRNA by real-time PCR (qPCR)***

191 cDNA concentration was assessed by UV-spectrophotometry (GeneQuant Pro) and 5 ng of each
192 sample were used as a template for real-time quantitative PCR (qPCR) performed by using StepOne
193 thermocycler (Applied Biosystems, StepOne software v. 2.1). The cDNA (5 ng/20 µL) was amplified
194 in triplicate with Fast SYBR Green Master Mix (Applied Biosystems) along with specific sets of
195 primers at 300 or 500 nM. The primers were designed based on published gene sequences or by using
196 Primer Express[®] software package (Applied Biosystems) to create oligonucleotides with similar
197 melting temperatures and minimal self-complementary, and purchased from Eurofins MWG Operon

198 (Ebersberg, Germany). Details of each primer set for detection of gene expression are reported in
199 Table 1.

200 The reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was selected as
201 endogenous control according to minimal intra-/inter-assay variation. Samples were kept at 95 °C for
202 20 s (hold step) to allow DNA-polymerase activation and then subjected to 40 cycles consisting of a
203 denaturation step at 95 °C for 3 s followed by an annealing/extension step at 60 °C for 30 s.
204 Fluorescence due to SYBR Green I incorporation was acquired at the end of the extension step. A
205 no-RT control and a no-template control (NTC) were included in each experiment. A melting curve
206 analysis for specific amplification control was performed (from 60 °C to 95 °C) at the end of the
207 amplification cycles.

208 NTC controls were assumed as negative and reliable if the quantification cycle (Cq) was ≥ 35 . Data
209 were analysed according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008)
210 in which expression levels of each gene, normalised to the GAPDH cDNA amount and expressed as
211 relative quantities to P5 as reference (RQ). The results of the expression of markers of differentiation
212 analysed in real-time PCR were also evaluated by calculating the ratio of: collagen II and collagen I,
213 aggrecan and versican, SMAD1/SMAD2 in the different step of subcultures.

214 **2.5 Statistical analysis**

215 The experimental results were reported as mean \pm standard error. Each experiment was performed in
216 triplicates. Normal distribution of data was verified by Kolmogorov-Smirnov test. Data were then
217 analysed with one-way analysis of variance (ANOVA) with subsequent Dunnett's multiple
218 comparison test (GraphPad Prism 7.0; La Jolla, CA). A p-value < 0.05 was considered statistically
219 significant. The statistical significance between the groups is plotted in the figures, whereas statistical
220 significance among subculture passages reported in the text.

221

222

223 **3. RESULTS**

224 **3.1 Morphology**

225 Immediately after seeding, freshly isolated chondrocytes (P0) had a rounded morphology that was
226 maintained within the first days of culturing (Fig. 1a). However, after 3-4 days of primary culture
227 (P1), the adherent cells became flattened and assumed an elongated fibroblastic-like shape or a
228 polygonal morphology. In association with cell adhesion and morphological changes, cells
229 proliferated and gradually formed a cell monolayer within 15 days and no differences were observed
230 between the three groups of animals. Going forward with the steps of primary culture (P1) (Fig. 1b
231 and c) and, particularly, in secondary culture (P2) (Fig. 1d), proliferating cells always appeared with
232 an elongated and polygonal morphology until they reach the confluence.

233 **3.2 Assessment of proliferative potential in adhesion culture by means of the CPDL**

234 The assessment of proliferation potential was performed with the calculation of the cell duplication,
235 known as CPDL, and mean values of CPDL obtained for each group of animals were reported in
236 relation to subculture passage (P) (Fig. 2). The chondrocyte growth of animals belonging to the
237 different groups showed a similar proliferative behavior up to 4th subculture (P4). After seeding, cells
238 continued to replicate with a constant trend until reaching CPDL value of 8 at P5. After that,
239 chondrocytes from animals of Group I (foals) and Group II (adults) achieved a maximum of 14
240 CPDL, even if chondrocytes from adult animals showed a significantly more rapid growth after
241 CPDL8 (P5), reaching the same CPDL as an earlier step (P7). Conversely, in animals over 12 years
242 of age (group III), the CPDL values at P6 and P7, were lower than those of group II; after that, an
243 evident reduction of proliferative capacity was observed. However, for chondrocytes belonging to
244 various groups after the 7th subculture (P7), it was noticed that there was a difficulty to adhere to the
245 surface and to furtherly proliferate at the same rate.

246

247 **3.3 Gene Expression of chondrocyte differentiation markers**

248 The gene expression analysis was carried out at P0, P1 and P3 because at P3 we obtained a large
249 number of chondrocytes and, as stated by others authors (Cheng et al., 2012; Ząbek et al., 2019), at
250 later passages chondrocytes were dedifferentiated as stated by gene expression markers.
251 Independent of the animal's age, PCR analysis showed that freshly isolated chondrocytes (P0) had a
252 high expression of some markers, namely Collagen type II (Col2), Aggrecan (ACAN), SOX9,
253 COMP, while the expression of collagen type I (Col1), Biglycan (BGN), RUNX2 was very low
254 (Fig. 3-6).

255 **3.3.1 Collagen type I , Collagen type II , Aggrecan and Versican gene expression**

256 In animals of group I (foals), P0 chondrocytes showed a significantly higher expression of Col2 and
257 ACAN compared to both the adult and elderly groups, together with a very low expression of Col1.
258 The gene expression of Col1 increased significantly ($p < 0,001$) from the P0 and reached the highest
259 levels at P3 subculture for the foal and adult groups (Fig. 3a). Common to all the groups, ACAN and
260 Col2 gene expression strongly decreased ($p < 0,001$) from P0 to P1 and P3 and both markers reached
261 very low levels at P3 subculture (Fig. 3 b-c). The same statistically significant gene expression trend
262 was observed for VCAN only in the adult and elderly groups, while the gene expression of VCAN in
263 foal P0 chondrocytes is significantly lower compared to the other two groups of animals, and it
264 maintained this lower trend without significant time related changes among sequential subculturing.
265 In particular, the ACAN showed a sudden decrease as early as in the primary culture, while the Col2
266 and, particularly the VCAN, showed a more gradual decline.
267 ACAN (Fig.3c) showed a significantly higher ($p < 0,001$) expression in P0 chondrocytes in young
268 animals compared to adults; the elderly had the lowest significant gene expression value. VCAN (Fig.
269 3d) gene expression in P0 chondrocytes was lower than the ACAN level; VCAN showed significantly
270 higher levels in elderly and adult animals than foals which showed very low starting levels.

271 **3.3.2 Cartilage Oligomeric Matrix Protein, Byglican, Fibromodulin and Collagen Type VI gene**
272 **expression.**

273 COMP expression in P0 chondrocytes showed significantly higher levels in foals, when compared to
274 other groups of animals (Fig. 4a); then it significantly decreased ($p<0.001$) for all groups from P0 to
275 P1 and P3, maintaining significantly higher levels in foals up to the P1 subculture.

276 Byglican gene expression in foals chondrocytes at P0 showed significantly higher levels than other
277 groups (Fig. 4b). Then, it significantly decreased for foals and elderly horses ($p<0.001$) after the first
278 subculture reached a gene expression without significant differences amongst each group.

279 Fibromodulin and Col6 in adult animals showed a significantly different trend than the other two
280 groups (Fig. 4 c, d). In adults, these markers of mature cartilage were significantly more expressed
281 from P0 ($p<0,001$) and maintained higher expression up to P3 ($p<0,05$) in comparison with foals and
282 elderly groups. Regarding the foals and the elderly, they both presented Fibromodulin and Col6 as
283 well as low gene expression between passages.

284

285 **3.3.3 SOX6, SOX9 and Runx2 gene expression**

286 The gene expression of SOX6 and SOX9 transcription factors showed a high expression in P0
287 chondrocytes; particularly, both SOX6 (Fig. 5a) and SOX9 (Fig. 5b) were expressed significantly
288 less in older animals whilst SOX9 already decreased in adults. During culturing, the SOX6 gene
289 expression in adult and young animals maintained significantly higher levels when compared to
290 elderly animals in subsequent subcultures, with a common slightly decreasing trend among groups
291 from P0 to P3. Instead SOX9 gene expression suddenly dropped ($p<0,001$) at P1 and then stabilised
292 at P3 subcultures without differences amongst the groups.

293 The expression of RUNX2 (Fig 5c), in elderly animals was significantly higher than the other groups
294 both in isolated chondrocytes and in the subcultures; presenting constant expression from P0 to P1
295 and a significant decrease ($p<0,05$) from P1 to P3. In foals and adults the P0 levels were low and then
296 gradually slightly increased with every culture passage.

297

298 **3.3.4 SMAD1, SMAD2 ALK5 and TGFβ1 gene expression**

299 The trends of SMAD2 and ALK5, (Fig 6 b, c) gene expression were similar in all three groups, with
300 significantly higher values in newly isolated chondrocytes, and a marked reduction ($p < 0,001$) after
301 the first subculture with a similar expression in subsequent culture passages. Regarding SMAD1, the
302 expression in freshly isolated chondrocytes from foals, were significantly lower ($p < 0,05$) than the
303 other two groups (Fig 6a); after an up and down trend this feature was also revealed at P3.

304 The trend of TGFβ1 gene expression showed that in adult animals, chondrocytes have significantly
305 higher levels in P0 up to P3 subculture, in comparison to the other two groups (Fig. 6d). In all groups,
306 TGFβ1 gene expression significantly decreased ($p < 0,001$) during subculturing, presenting the highest
307 decrease passing from P0 to P1.

308

309 **3.3.5 Coll II/Coll I, Aggrecan/Versican and SMAD1/SMAD2 ratios**

310 The ratios declined during the subsequent subculture passages.

311 In particular, the Col2/ Coll1 ratio (Fig. 7a) significantly decreased ($p < 0,001$) from the very first
312 culture passage with a further decrease in the subsequent culturing steps, progressively reaching the
313 lowest levels. This ratio in P0 chondrocytes was significantly different among the three groups with
314 the highest value in foals, intermediate in adults and lowest in the elderly, without any differences
315 amongst the groups in the subsequent passages. The decrease of the Col2/Coll1 ratio was quick in
316 foals and adult groups reaching basal value at P3.

317 ACAN/VCAN ratio (Fig. 7b) showed a higher value in foals, mostly due to the initial higher levels
318 of ACAN in this group, followed by adult and elderly. The ACAN/VCAN ratios showed a significant
319 downward trend ($p < 0,001$ adults and foals; $p < 0,05$ elderly) in all three groups, reaching values close
320 to minimum at P3 subculture in the elderly group.

321 The SMAD1/SMAD2 ratio (Fig. 4c) in freshly isolated chondrocytes was higher in young animals
322 than adults and elderly. Only for the foal group did the ratio significantly decrease ($p<0,001$) from P0
323 to P1 and P3, while for the other groups it remained constant among subcultures.

324

325 4. DISCUSSION

326 “Matrix-associated Chondrocyte Implantation” (MACI), shows an intrinsic problem linked to the
327 progressive dedifferentiation of adult chondrocytes, a condition that reduces or loses their capacity to
328 form cartilage *in vivo* after implantation (Ma et al., 2013). Also, despite the current improvement of
329 type and structure of biomaterials used in MACI, the process of re-differentiation sometimes remains
330 incomplete (Albrecht et al., 2011; Goldberg, Lee, Bader, & Bentley, 2005; Jakob et al., 2001).

331 Therefore considering the increasing interest in tissue engineering, new chondroprotective agents and
332 cell-based therapies for OA in horses were clinically applied (Cokelaere, Malda, & van Weeren, 2016;
333 Suchorska, Augustyniak, Richter, & Trzeciak, 2017).

334 It is also very important to identify the molecular markers suitable for monitoring the de-
335 differentiation changes *in vitro* over time, before constructs are implanted *in vivo*, as well as taking
336 into consideration the influence of age on donors.

337 In this work, the Cumulative Population Doubling Level (CPDL) (M. S. Seo et al., 2013) is used as
338 a parameter to evaluate the proliferative potential of chondrocytes in culture adhesion, whilst the
339 passage (P) number refers to the number of times chondrocytes are serially passaged in monolayer.
340 It is worth noting that the CPDL expansion factor does not correlate with the passage number. This
341 is important because, when biomaterials are used for tissue engineering, should be indicated in detail
342 the suitable passage number as well as the information on the cell expansion factor (Huang, Hu, &
343 Athanasiou, 2016).

344 After a linear proliferation phase, during the final subculturing steps, we demonstrated that adult horse
345 chondrocytes have a reduced adhesion and proliferative capacity due to cellular senescence *in vitro*

346 and due to the age of the donor animals (Jeong, Lee, & Kim, 2014). The age of chondrocytes limits
347 the chondrocyte proliferation and also significant differences on the proliferative potential are related
348 to the age of chosen animals: chondrocytes from elderly animals lack proliferate beyond the CPDL8
349 (P6 in culture) and, optically, a reduced ability to adhere to a surface was observed.

350 In accordance with our results, several studies, in other models, showed that age is an important
351 contributing factor to impaired efficacy of cartilage repair (Toh et al., 2016).

352 Horse articular chondrocytes, maintain a very high proliferative activity *in vitro*, as well as
353 morphological changes undergo functional modifications linked to a progressive state of
354 dedifferentiation altering their gene expression from typical ECM components to expressing genes
355 typical of a pre-chondrogenic mesenchymal or hypertrophic status (Oldershaw, 2012) and we
356 observed these changes from just after isolation up to the first subculture.

357 The trend of ECM genes showed a similar decrease over time in the three groups; however, the values
358 of isolated chondrocytes and the extent of variation were different.

359 Freshly isolated chondrocytes typically express very high levels of Col2 and Aggrecan major matrix
360 molecules of mature articular cartilage (Binette et al., 1998; Kiani, Chen, Wu, Yee, & Yang, 2002;
361 Schulz & Bader, 2007) and SOX-9, the nuclear transcription factor essential for the synthesis of Col2
362 and Aggrecan (X. Li, Leo, Beck, Balian, & Anderson, 2004) and a potent inhibitor the chondrocyte
363 hypertrophy (G. Liu et al., 2007; Ma et al., 2013). During the expansion phase in culture, a strong
364 reduction of these typical cartilage markers occurred immediately at P1 (Darling & Athanasiou,
365 2005), while the levels of Col I increased. These results are in partial agreement with other authors
366 that have found a progressive decrease of Coll2 until the end of culture (Diaz-Romero, Nestic,
367 Grogan, Heini, & Mainil-Varlet, 2008).

368 These molecular changes vary with the age of the donor, especially in chondrocytes obtained from
369 young animals where Col2 gene expression undergoes a drastic reduction.

370 Collagen I is a major marker of de-differentiation (Semevolos et al., 2001). Immediately after
371 isolation, chondrocytes from young and adults animals do not express collagen I exactly as the elderly

372 animals. However, after the first step in culture, Col1 expression also significantly increased in foals,
373 where earlier it reached higher values than the other groups.

374 In chondrocytes from elderly subjects, Col I had the highest expression from the very first passage of
375 culture. This indicates that in older animals chondrocytes only undergo a modification of their
376 chondrocytic phenotype in the native tissue (Garvican, Vaughan-Thomas, Redmond, & Clegg, 2008)
377 since Collagen I was expressed earlier and in greater quantities.

378 In these animals, the level of dedifferentiation was extremely reduced at P6-P7, where lower values
379 of CPDL were also noticed. The Collagen I expression occurs early (in the first passage in culture)
380 and differentiation markers, such as collagen II, ACAN and SOX9, are drastically reduced within
381 P1. This indicates that, in these animals, the *in vitro* amplification may not produce a suitable quantity
382 of cells for transplantation neither as a number nor as a differentiation potential.

383 VCAN was highly expressed during early cartilage development, then rapidly decreased during
384 growth, being expressed at low levels in mature articular cartilage suggesting a primary role in the
385 development of cartilage, but a minor role in maintenance of phenotype (Kimata et al., 1986;
386 Shinomura, Jensen, Yamagata, Kimata, & Solursh, 1990).

387 Versican had relatively low expression levels in P0 chondrocytes from foals when compared to adult
388 and old animals. It then decreased during subculturing in all groups when dedifferentiation occurred.
389 Other studies supported a diversified and wide role of versican (Wight 2002) indeed it is also present
390 in mature cartilage where it interacts with the cells and with other ECM molecules to regulate the
391 microenvironment that supports hyaline cartilage formation. (Taylor et al., 2014).

392 We have confirmed that ratios between some markers can be also used as indicators of the level of
393 differentiation/dedifferentiation (Martin et al., 2001). The ratios between Col II/Col I and
394 ACAN/VCAN (Wight, 2002) decrease during subculturing *in vitro*.

395 The Col2/Col1 ratio had very high values in P0 chondrocytes of foals, a lower value in adults and the
396 lowest in elderly subjects, suggesting that the three groups of donors have a differentiation potential
397 that differs just from the onset of *in vitro* culture.

398 The ACAN/VCAN ratio seems a more probative parameter of dedifferentiation when compared to
399 the single gene markers considered. The shifting balance of the ACAN/VCAN ratio in favor of
400 VCAN indicates a progressive loss of differentiation at P1 which is even more pronounced in animals
401 over 12 years at P3 subculture.

402 Moreover, biglycan (BGN), fibromodulin (FMOD), Cartilage Oligomeric Matrix Protein (COMP and
403 collagen 6 (Coll6) , macromolecules of mature matrix involved in maintaining the integrity of articular
404 cartilage, were studied.

405 Byglican and Fibromodulin belong to the family of SLRPs (Small Leucine Rich Proteoglycans)
406 present in articular cartilage.

407 Byglican participates in assembling the cartilage extracellular matrix, by interacting with Collagen
408 VI and Large Aggregating Proteoglycans (LAPs); it may influence chondrocyte differentiation by
409 regulating the expression of EGF receptors (Jakob et al., 2001).

410 Fibromodulin (FMOD) is a small leucine-rich proteoglycans that is expressed at sites of cartilage
411 formation during development (Wilda et al., 2000). FMOD helps to maintain the integrity of adult
412 articular cartilage and modulates its metabolism; it plays a role in collagen fibrillogenesis binding to
413 the collagen II fibrils, to regulates their diameter promoting fibril-fibril interactions in the matrix
414 (Jepsen et al., 2002). FMOD also interacts with many other macromolecules, including collagen types
415 VI and XIV, elastin, fibronectin and growth factors (such as TGF β , EGF and TNF α) (Embree et al.,
416 2010).

417 The Cartilage Oligomeric Matrix Protein (COMP) belongs to the thrombospondine family. Its main
418 role is to stimulate the formation of Collagen II fibrils and it's involved both in early chondrogenesis
419 and in mature cartilage interacting with other molecules of ECM. Particularly, during cartilage
420 turnover, COMP is cleaved and its fragments are released into synovial fluid and used as markers of
421 joint pathologies (Roughley, 2001).

422 Byglican and COMP are more highly expressed in chondrocytes isolated from young P0 but, their
423 behavior differs during *in vitro* expansion: byglican undergoes a rapid decrease already at P1, while
424 COMP gene expression is maintained longer during culturing.

425 The different trends of these two early markers of chondrogenesis, demonstrates that dedifferentiation
426 has a lower influence on COMP gene expression, showing that it is a more stable marker of
427 chondrocyte phenotype in young cartilage (Zaucke, Dinser, Maurer, & Paulsson, 2001).

428 Collagen VI and Fibromodulin were more highly expressed in adult animals and their expression
429 progressively reduced throughout the culture passages.

430 The presence of these markers is reduced in young subjects since the structure of ECM in these
431 animals is not yet totally mature and therefore the interaction between extracellular macromolecules
432 is not completed.

433 Conversely, the reduced expression in elderly animals, could be due to age. Specifically, it has been
434 observed that the age-dependent reduction in the synthesis of chondroitin sulfate chains (being part of
435 the fibromodulin structure) may result in the accumulation of fibromodulin in the non-glycated form
436 in the cartilage of adult subjects compared to younger subjects (Roughley, 2001).

437 In adult animals, Col6 and FMOD could more specifically indicate a stable differentiation status
438 since the ability to synthesise characteristic molecular components of the cartilage matrix is lost.

439 Particularly, Col VI is an important component of the ECM of mature articular cartilage, mainly
440 concentrated in the pericellular matrix, where is involved in the attachment and integrity of
441 chondrocytes (Cescon, Gattazzo, Chen, & Bonaldo, 2015). In this location, Col VI has a role in the
442 transmission of mechanical and physico-chemical signals from the ECM to the pericellular matrix
443 (Zelenski et al., 2015). Furthermore, Smeriglio et al have shown that Col VI is an important stimulus
444 for the proliferation of adult chondrocytes, suggesting that it can be used for the expansion of
445 chondrocytes, such as in autologous chondrocyte transplantation or in tissue-engineering applications
446 (Smeriglio et al., 2015).

447 Also transcription factors as SOX6/SOX9 and RUNX2 change during dedifferentiation. Members of
448 the SOX transcription factor, namely SOX9 in association with SOX5 and SOX6, are required for
449 chondrogenesis (Akiyama, Chaboissier, Martin, Schedl, & De Crombrughe, 2002; Ikeda et al., 2004)
450 and have a critical role in the early stages of chondrogenesis until they reach the chondrocyte
451 hypertrophy. Research data has suggested that SOX9 and L-SOX5/SOX6 cooperate with each other
452 to directly activate Col2 synthesis (Lefebvre & Smits, 2005).

453 The reduction of SOX9 and SOX6 expression in all three groups confirms a process of chondrocyte
454 de-differentiation of cultured chondrocytes (Caron et al., 2012; Cournil-Henrionnet et al., 2008),
455 furtherly defined by the appearance of not-dominant RUNX2 gene expression (Zhou et al., 2006).
456 Moreover, a reduced level of SOX6 in chondrocytes of elderly horses even supports the hypothesis
457 that in these animals the chondrogenic capacity is significantly reduced after the first two weeks of
458 culture. Runx2 is a transcription factor positively regulated during the progression of the chondrocyte
459 towards a hypertrophic phenotype (Takeda, Bonnamy, Owen, Ducy, & Karsenty, 2001) and is
460 normally expressed during endochondral ossification in addition to Ihh, Col10 and BMP-6 . Thus,
461 unlike SOX9, Runx2 it's generally considered a major driver for the later stages of endochondral
462 ossification, up-regulating the synthesis of collagen type 1, typical of the bone matrix, and of MMP-
463 13, which is involved in the proteoglycans degradation and calcification process (Chen et al., 2014).
464 Runx2 expression has shown an opposite trend in elderly animals in comparison to other groups,
465 having already high expression already high in freshly isolated chondrocytes (P0) while in the other
466 two groups appears after the P3. This could furtherly indicate a more advanced dedifferentiation status
467 in these individuals than foals and adults (Caron et al., 2012). In addition, the increased levels of
468 RUNX2 could indicate limited of recovery after this threshold.

469 Several growth factors affect the chondrocytes metabolism, in particular we noted TGF- β 1 that
470 activates the Smad2/3 signaling pathway, stimulating expression of type II collagen α 1, aggrecan and
471 sex determining region Y-box 9 expression in human OA and bovine chondrocytes (Zhu et al., 2015).
472 The decreasing trend of TGF- β 1, an anabolic factor for articular chondrocytes which stimulates them

473 to release extracellular matrix molecules (Cheng et al., 2012), supports the hypothesis that P3
474 subculture is a critical step for full chondrocyte dedifferentiation in monolayer expansion.
475 The study analysed, for the first time the chondrogenic pathway during different proliferative steps
476 *in vitro* of horse articular chondrocytes. Precisely, an extended pattern of gene expression was
477 analysed in relation to cartilage matrix components, with the aim to identify markers that may
478 distinguish differentiated chondrocytes from dedifferentiated chondrocytes, and to furtherly
479 characterise chondrocyte dedifferentiation.

480 Over all, the present work demonstrated that chondrocyte dedifferentiation started early in culture
481 (P0-P1), was very marked at P3 subculture (CPDL 4-6), which appeared critical for maintenance/loss
482 of differentiation potential and this is most evident in older subjects (Kang, Yoo, & Kim, 2007).
483 When de-differentiation occurs, the expression of COL2A1 and ACAN was lost, while the expression
484 of COL1A1 increased (Benya, Padilla, & Nimni, 1978; Mayne, Vail, Mayne, & Miller, 1976; Von
485 Der Mark, Gauss, Von Der Mark, & Müller, 1977). The drastic reduction of all chondrogenic markers
486 from newly isolated chondrocytes to proliferating chondrocytes in adhesion culture confirms that the
487 *in vitro* expansion phase is a highly critical stage for maintaining the differentiation of chondrocytes
488 with very low gene levels compared to mature chondrocytes in native tissue.

489 Chondrocytes harvested from elderly horses showed a partial dedifferentiation immediately after
490 isolation from the cartilage with occurred very early in culture, associated with a reduced proliferative
491 capacity.

492 Nevertheless, until the P3 passage, some markers of mature cartilage, (Col6, FMOD, SOX6) are
493 preserved. Specifically, chondrocytes from adult donors showed a more stable expression of Col6,
494 Fibromodulin, SOX6, TGF β 1. The associated trend of Col6, fibromodulin, SOX6 and TGF β 1
495 regarding changes of Runx2 testifies that these markers could be the most suitable to monitor the
496 dedifferentiation process.

497 **CONCLUSIONS**

498 In conclusion, this study has provided the identification of molecular markers suitable for monitoring
499 the level of dedifferentiation beyond which chondrocytes are no longer able to have a recovery
500 capacity and, consequently, which "culture step" is sufficient to obtain the correct number of
501 chondrocytes for transplantation with a phenotypic potency as conserved as possible based on the
502 donor age. To confirm which culture passage is critical for differentiation, ongoing studies should be
503 conducted using dedifferentiated chondrocyte at different passages (P or PDL), then cultured on
504 scaffolds or a 3D systems that can support chondrocyte re-differentiation (Caron et al., 2012).
505 With this perspective, it will be possible to obtain expanded chondrocytes that potentially maintain a
506 differentiated status, able to be used in *in vivo* implants for tissue engineering or as model *in vitro* to
507 evaluate new three-dimensional systems or medium components able to maintain differentiation or
508 to re-differentiate articular chondrocytes. Similarly, the identification of differentiation markers and
509 their culture-related changes, are equally important in the characterisation of the chondrogenetic
510 process starting with different types of stem cells (K. F. Ortved & Nixon, 2016).

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513 **CONFLICT OF INTEREST STATEMENT**

514 The authors certify that there is no actual or potential conflict of interest in relation to this article.

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Gene	Primer sequences	Primers concentration (nM)
GAPDH	FWD: CAAGGCTGTGGGCAAGGT REV: GGAAGGCCATGCCAGTGA	300 300
ACAN	FWD: GACCACTTTACTCTTGCGTTTG REV: GTCAGGGTCTGAAACGTCTACTGA	500 500
SOX9	FWD: CAGGTGCTCAAGGGCTACGA REV: GACGTGAGGCTTGTCTTGCT	300 300
COL2A1	FWD: CTGGTGATGATGGTGAAG REV: GTAACCTCTGTGACCTTTG	300 300
COL1A1	FWD: AGAAGAAGACATCCCAGCAGTCA REV: CAGGGCTCGGGTTCCATA	500 500
BGN	FWD: CTGGACCTGCAGAACAATGAGA REV: CAGGACGAGGGCATAGAGATG	300 300
COMP	FWD: GAGATCGTGCAAACAATGAACAG REV: GCCATTGAAGGCCGTGTAAC	300 300
RUNX2	FWD: CCCGTGGCCTTCAAAGTG REV: TGACAGTAACCACAGTCCCATCTG	300 300
COL6A2	FWD: CACCATCAACCGCATCATCA REV: TCCAGACAGCTCACTTTGTAGCA	300 300
VCAN	FWD: GCAACCCATGCACTACATAAAGTC REV: TCCAGAGAGGGAGCCCTTAAC	500 500
SOX6	FWD: TGGCTGAAGCGCGAGTCTA REV: CCTTCGCCCAAACCATGA	300 300
ALK5	FWD: CGCGAGAACTATCGTGTTACAAGA REV: CTGCACTTGGCTGGCAAA	300 300
TGFβ1	FWD: CCGAGCTCTGGACACCAACTAC REV: TGCCGTACGCAGCAGTTC	300 300
SMAD1	FWD: CAATGCCACTTTTCCGGATT REV: GGGAGTGAGGAAACGGATGA	300 300
SMAD2	FWD: CCAACGTCAACCGAAATGC REV: AGCGCACTCCTCTCCCTATATG	300 300
FMOD	FWD: AATGGCTCAGGCTTCAAAGAG REV: CCACTACGGATGCTGATGATCA	300 300

526 **Table1.** Primer sequences used for real-time polymerase chain reaction **GAPDH** - glyceraldehyde-
527 3-phosphate dehydrogenase; **ACAN** – Aggrecan; **COL2A1** - Collagen type II; – **COL1A1** Collagen
528 type I – **BGN** – Byglican; **COMP** – Cartilage Oligomeric Matrix Protein; **RUNX2** – Runt-related

529 transcription factor 2; **COL6A2** – Collagen Type VI; **VCAN** – Versican; **SOX6** – Transcription
530 factor SOX-6; **ALK5**- TGF β type I receptor kinase; **TGF β 1** - Transforming growth factor beta
531 receptor I; **SMAD1** – **SMAD2**- small mother against decapentaplegic 1 and 2; **FMOD** -
532 Fibromodulin.

533

534 **Figure caption**

535

536 **Figure 1.** Optical microscopy of freshly isolated chondrocytes after 1 day (A), 5 days (B) and 10
537 days (C) of primary culture; in fig. D a secondary culture.

538 **Figure 2.** Mean values and standard deviations of CPDL of cultured chondrocytes obtained from
539 animals of the three different groups. Statistical differences within a group over time are indicated
540 with an asterisk (*).

541

542 **Figure 3.** Mean values of gene expression of Collagen Type I (Col1) (A), Collagen Type II (Col2)
543 (B), Aggrecan (ACAN) (C) and Versican (VCAN) (D) in freshly isolated chondrocytes (P0) and
544 subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly).
545 Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and
546 elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no statistical differences
547 among the three groups.

548

549 **Figure 4.** Mean values of gene expression of Cartilage Oligomeric Matrix Protein (COMP) (A),
550 Byglican (BGN) (B), Fibromodulin (FMOD) (C), Collagen Type VI (Col6) (D) in freshly isolated
551 chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals,
552 adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups
553 (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no
554 statistical differences among the three groups.

555

556 **Figure 5.** Mean values of gene expression of SOX6 (A), SOX9 (B) and Runx2 (C) in freshly isolated
557 chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals,
558 adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups

559 (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no
560 statistical differences among the three groups.

561

562 **Figure 6.** Mean values of gene expression of and SMAD1 (A), SMAD2 (B), ALK5 (C) and TGF β 1
563 (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-
564 dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant
565 differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while
566 no letters indicates no statistical differences among the three groups.

567

568 **Figure 7.** Mean values of gene expression calculated as ratios between Collagen II/Collagen I (A),
569 Aggrecan/Versican (B) and SMAD2/SMAD1(C) in freshly isolated chondrocytes (P0) and
570 subculturing (P1,P3) from animals of different age-dependent group (foals, adult and elderly).
571 Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and
572 elderly) at each subculturing passage (P0,P1,P3); while no letters indicates no statistical differences
573 among the three groups.

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576 We thank Dr. Martina Campo and Dr. Francesca Beretta for their technical collaboration.

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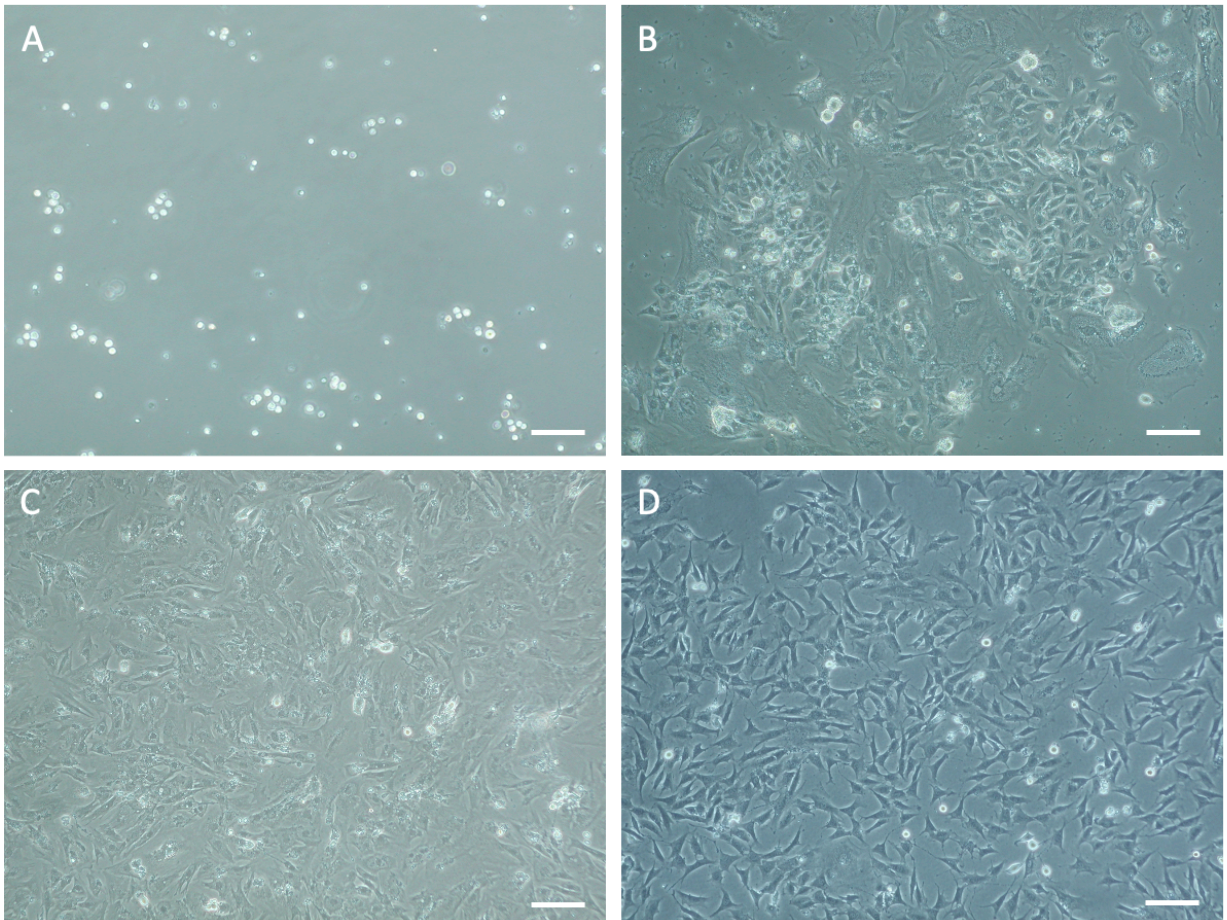


Fig. 1. Optical microscopy of freshly isolated chondrocytes after 1 day (A), 5 days (B) and 10 days (C) of primary culture; in fig. D a secondary culture.

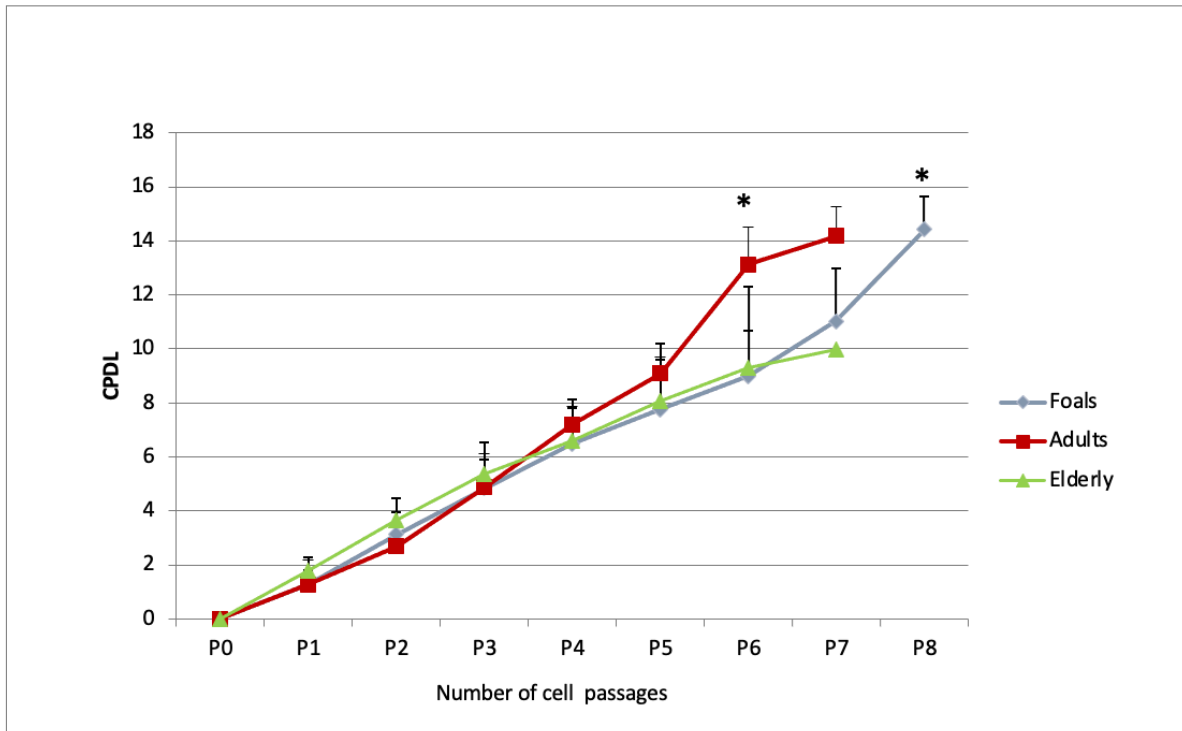


Fig. 2. Mean values and standard deviations of CPDL of cultured chondrocytes obtained from animals of the three different groups. Statistical differences within a group over time are indicated with an asterisk (*).

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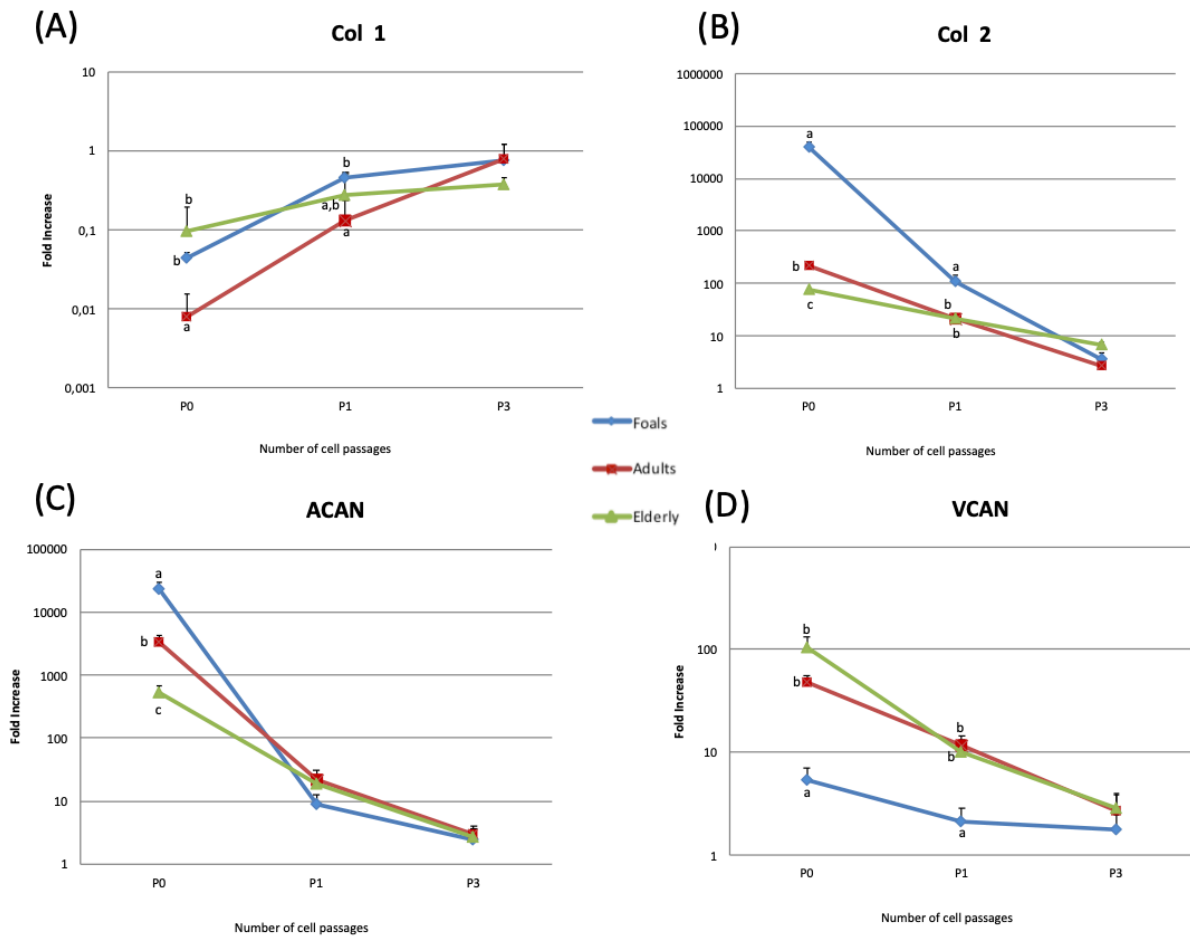


Fig. 3. Mean values of gene expression of Collagen Type I (Col1) (A), Collagen Type II (Col2) (B), Aggrecan (ACAN) (C) and Versican (VCAN) (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no statistical differences among the three groups.

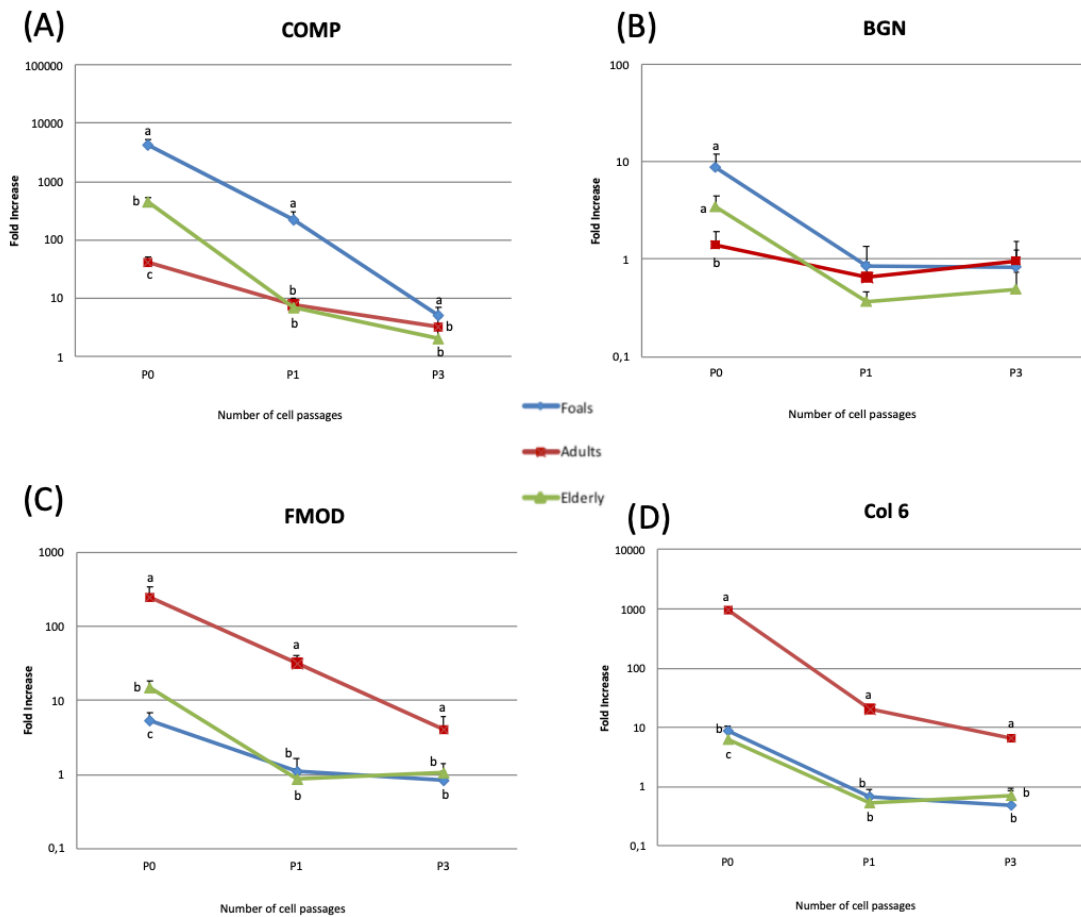


Fig. 4. Mean values of gene expression of Cartilage Oligomeric Matrix Protein (COMP) (A), Byglican (BGN) (B), Fibromodulin (FMOD) (C), Collagen Type VI (Col6) (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no statistical differences among the three groups.

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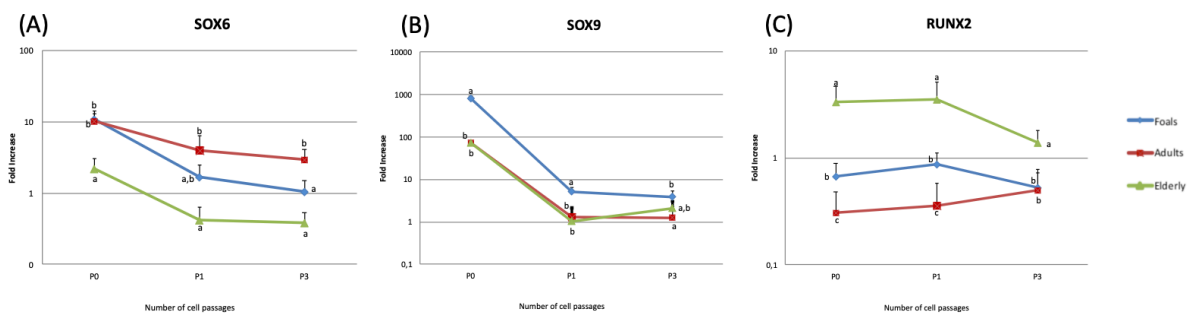


Fig. 5. Mean values of gene expression of SOX6 (A), SOX9 (B) and Runx2 (C) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicate no statistical differences among the three groups.

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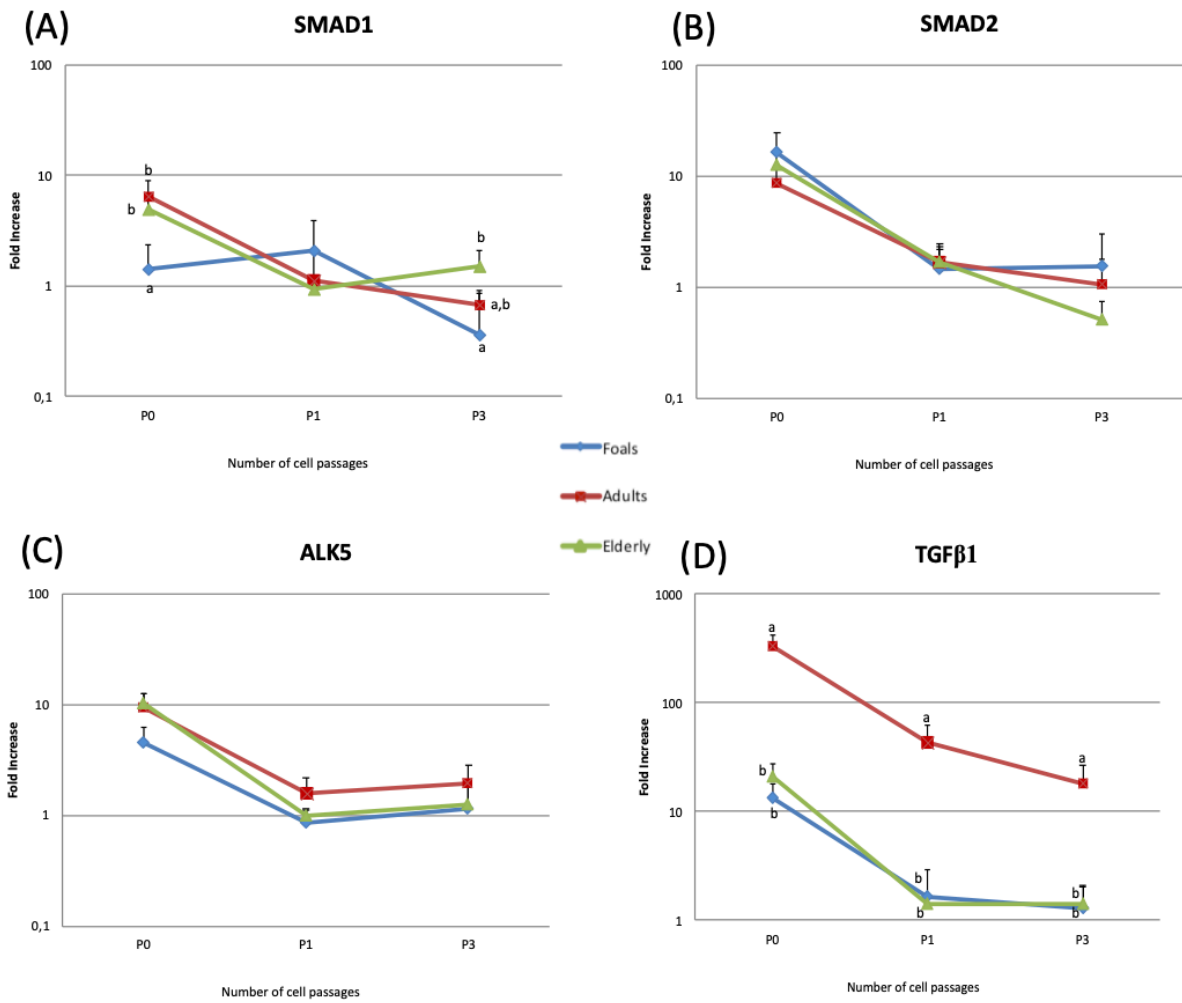


Fig. 6. Mean values of gene expression of SMAD1 (A), SMAD2 (B), ALK5 (C) and TGFβ1 (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicates no statistical differences among the three groups.

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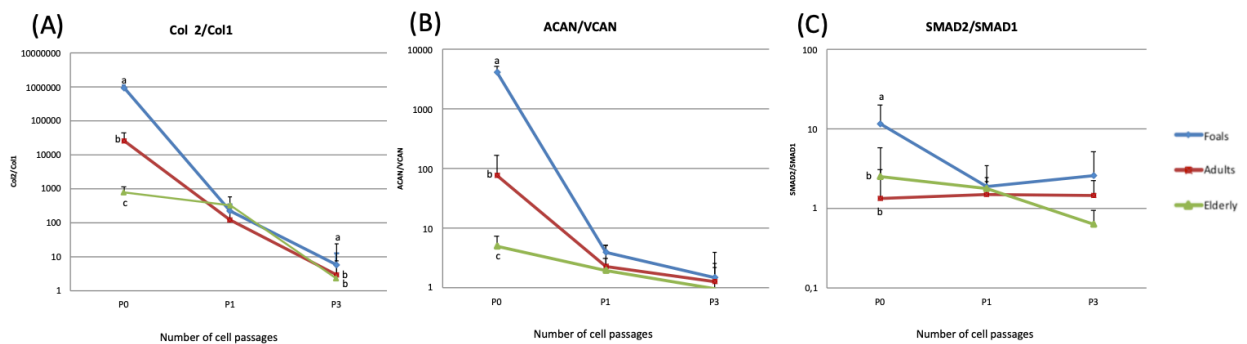


Fig. 7. Mean values of gene expression calculated as ratios between Collagen II/Collagen I (A), Aggrecan/Versican (B) and SMAD2/SMAD1(C) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent group (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups (foals, adult and elderly) at each subculturing passage (P0,P1,P3); while no letters indicates no statistical differences among the three groups.

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