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

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

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Abstract

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

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

1. INTRODUCTION

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

Indeed, once damaged, the articular cartilage is unable to heal spontaneously and the repair process leads to the formation of a fibro-cartilaginous tissue which does not have the native mechanical properties of hyaline cartilage (Goldring & Goldring, 2007; McIlwraith, Frisbie, & Kawcak, 2012). Furthermore, the increased age of animals is an important risk factor of OA because the senescence of chondrocytes results in the development of secretory phenotype and ageing changes in the matrix (Rahmati, Nalesso, Mobasheri, & Mozafari, 2017; Shane Anderson & Loeser, 2010).

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

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

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

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

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

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

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

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

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

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

Since the mechanisms underlying the dedifferentiation process in articular chondrocytes were poorly studied in horses, we evaluated an extended pattern of gene expression related to cartilage matrix components, aiming to identify markers that may distinguish articular differentiated chondrocytes from dedifferentiated chondrocytes, and to finally characterise chondrocyte dedifferentiation. Aggrecan (ACAN) and Collagen Type II (COL2A1) are the major matrix molecules of mature articular cartilage (Schulz & Bader, 2007) while Collagen I is the largest fibrillar collagen produced by osteoblasts and its expression was observed in joint disorders characterised by an extracellular matrix disorganisation and by the assumption of a typical fibroblast-like phenotype (Semevolos, Nixon, & Brower-Toland, 2001). Other small matrix molecules such as Collagen Type VI (Coll6), Biglycan (BGN), Fibromodulin (FMOD) and Cartilage Oligomeric Matrix Protein (COMP) are mainly involved in the interaction with other macromolecules of mature matrix to maintain the integrity of articular cartilage (Wilda et al. 2000). SOX6/SOX9 and RUNX2 encodes transcription factors, SOX6/SOX9 are essential for early chondrocyte differentiation (Li, Leo, Beck, Balian, & Anderson, 2004) and RUNX 2 for expression of genes is mainly relevant for the development of hypertrophic and terminal chondrocytes (C. F. Liu, Samsa, Zhou, & Lefebvre, 2017). Moreover TGF-β1 is an anabolic factor for articular chondrocytes that acts through the ALK5/SMAD2 pathway, 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

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

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

Freshly isolated chondrocytes (P0: Passage 0) were plated (2 x 10^4 cells/cm^2) in triplicate in a Petri dish (57cm^2) (Sarstedt, Nümbrecht, Germany) cultured in D-MEM with 4,5 g/l glucose (SIGMA Aldrich) with 10% FCS at 37°C and 5%CO_2. The medium was changed every three days. The cultures (P1: Passage 1) were observed by phase contrast microscopy at 1, 5 and 10 days of culture. After reaching monolayer, chondrocytes were detached with 0,25% trypsin – 0,02% EDTA (SIGMA Aldrich) and counted using a Bürker hemocytometer; then 2 × 10^4 cells were reseeded in a secondary subculture (P2: Passage 2); the other subcultures (P3, P4, P5 ecc.) were set up until the cells stopped proliferating.

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

2.4 Gene Expression of Differentiation Markers

2.4.1 Total RNA Extraction and cDNA Synthesis.

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

2.4.2 Reverse-transcription (RT)

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

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

cDNA concentration was assessed by UV-spectrophotometry (GeneQuant Pro) and 5 ng of each sample were used as a template for real-time quantitative PCR (qPCR) performed by using StepOne thermocycler (Applied Biosystems, StepOne software v. 2.1). The cDNA (5 ng/20 μL) was amplified in triplicate with Fast SYBR Green Master Mix (Applied Biosystems) along with specific sets of primers at 300 or 500 nM. The primers were designed based on published gene sequences or by using Primer Express® software package (Applied Biosystems) to create oligonucleotides with similar 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 $2^{-\Delta\Delta Ct}$ 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 ± 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

3. RESULTS

3.1 Morphology

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

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

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

3.3 Gene Expression of chondrocyte differentiation markers

The gene expression analysis was carried out at P0, P1 and P3 because at P3 we obtained a large number of chondrocytes and, as stated by others authors (Cheng et al., 2012; Ząbek et al., 2019), at later passages chondrocytes were dedifferentiated as stated by gene expression markers.

Independent of the animal's age, PCR analysis showed that freshly isolated chondrocytes (P0) had a high expression of some markers, namely Collagen type II (Col2), Aggrecan (ACAN), SOX9, COMP, while the expression of collagen type I (Col1), Byglican (BGN), RUNX2 was very low (Fig. 3-6).

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

In animals of group I (foals), P0 chondrocytes showed a significantly higher expression of Col2 and ACAN compared to both the adult and elderly groups, together with a very low expression of Col1. The gene expression of Col1 increased significantly ($p<0,001$) from the P0 and reached the highest levels at P3 subculture for the foal and adult groups (Fig. 3a). Common to all the groups, ACAN and Col2 gene expression strongly decreased ($p<0,001$) from P0 to P1 and P3 and both markers reached very low levels at P3 subculture (Fig. 3 b-c). The same statistically significant gene expression trend was observed for VCAN only in the adult and elderly groups, while the gene expression of VCAN in foal P0 chondrocytes is significantly lower compared to the other two groups of animals, and it maintained this lower trend without significant time related changes among sequential subculturing. In particular, the ACAN showed a sudden decrease as early as in the primary culture, while the Col2 and, particularly the VCAN, showed a more gradual decline.

ACAN (Fig.3c) showed a significantly higher ($p<0,001$) expression in P0 chondrocytes in young animals compared to adults; the elderly had the lowest significant gene expression value. VCAN (Fig. 3d) gene expression in P0 chondrocytes was lower than the ACAN level; VCAN showed significantly higher levels in elderly and adult animals than foals which showed very low starting levels.

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

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

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

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

3.3.3 SOX6, SOX9and Runx2 gene expression

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

The expression of RUNX2 (Fig 5c), in elderly animals was significantly higher than the other groups both in isolated chondrocytes and in the subcultures; presenting constant expression from P0 to P1 and a significant decrease ($p<0,05$) from P1 to P3. In foals and adults the P0 levels were low and then 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/ Col1 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/Col1 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.

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

4. DISCUSSION

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

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

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

In this work, the Cumulative Population Doubling Level (CPDL) (M. S. Seo et al., 2013) is used as a parameter to evaluate the proliferative potential of chondrocytes in culture adhesion, whilst the passage (P) number refers to the number of times chondrocytes are serially passaged in monolayer. It is worth nothing that the CPDL expansion factor does not correlate with the passage number. This is important because, when biomaterials are used for tissue engineering, should be indicated in detail the suitable passage number as well as the information on the cell expansion factor (Huang, Hu, & Athanasiou, 2016).

After a linear proliferation phase, during the final subculturing steps, we demonstrated that adult horse 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, Nesic,
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 occured.
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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Several growth factors affect the chondrocytes metabolism, in particular we noted TGF-β1 that activates the Smad2/3 signaling pathway, stimulating expression of type II collagen α1, aggrecan and sex determining region Y-box 9 expression in human OA and bovine chondrocytes (Zhu et al., 2015). The decreasing trend of TGF-β1, an anabolic factor for articular chondrocytes which stimulates them

to release extracellular matrix molecules (Cheng et al., 2012), supports the hypothesis that P3 subculture is a critical step for full chondrocyte dedifferentiation in monolayer expansion.

The study analysed, for the first time the chondrogenic pathway during different proliferative steps *in vitro* of horse articular chondrocytes. Precisely, an extended pattern of gene expression was analysed in relation to cartilage matrix components, with the aim to identify markers that may distinguish differentiated chondrocytes from dedifferentiated chondrocytes, and to furtherly characterise chondrocyte dedifferentiation.

Over all, the present work demonstrated that chondrocyte dedifferentiation started early in culture (P0-P1), was very marked at P3 subculture (CPDL 4-6), which appeared critical for maintenance/loss of differentiation potential and this is most evident in older subjects (Kang, Yoo, & Kim, 2007).

When de-differentiation occurs, the expression of COL2A1 and ACAN was lost, while the expression of COL1A1 increased (Benya, Padilla, & Nimni, 1978; Mayne, Vail, Mayne, & Miller, 1976; Von Der Mark, Gauss, Von Der Mark, & Müller, 1977). The drastic reduction of all chondrogenic markers from newly isolated chondrocytes to proliferating chondrocytes in adhesion culture confirms that the *in vitro* expansion phase is a highly critical stage for maintaining the differentiation of chondrocytes with very low gene levels compared to mature chondrocytes in native tissue.

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

Nevertheless, until the P3 passage, some markers of mature cartilage, (Col6, FMOD, SOX6) are preserved. Specifically, chondrocytes from adult donors showed a more stable expression of Col6, Fibromodulin, SOX6, TGFβ1. The associated trend of Col6, fibromodulin, SOX6 and TGFβ1 regarding changes of Runx2 testifies that these markers could be the most suitable to monitor the dedifferentiation process.

CONCLUSIONS

In conclusion, this study has provided the identification of molecular markers suitable for monitoring the level of dedifferentiation beyond which chondrocytes are no longer able to have a recovery capacity and, consequently, which "culture step" is sufficient to obtain the correct number of chondrocytes for transplantation with a phenotypic potency as conserved as possible based on the donor age. To confirm which culture passage is critical for differentiation, ongoing studies should be conducted using dedifferentiated chondrocyte at different passages (P or PDL), then cultured on scaffolds or a 3D systems that can support chondrocyte re-differentiation (Caron et al., 2012).

With this perspective, it will be possible to obtain expanded chondrocytes that potentially maintain a differentiated status, able to be used in *in vivo* implants for tissue engineering or as model *in vitro* to evaluate new three-dimensional systems or medium components able to maintain differentiation or to re-differentiate articular chondrocytes. Similarly, the identification of differentiation markers and their culture-related changes, are equally important in the characterisation of the chondrogenetic process starting with different types of stem cells (K. F. Ortved & Nixon, 2016).

CONFLICT OF INTEREST STATEMENT

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

Table 1

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

Table1. Primer sequences used for real-time polymerase chain reaction **GAPDH** - glyceraldehyde-3-phosphate dehydrogenase; **ACAN** – Aggrecan; **COL2A1** - Collagen type II; – **COL1A1** Collagen 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**- TGFβ type I receptor kinase; **TGFβ1** - Transforming growth factor beta
531 receptor I; **SMAD1** – **SMAD2**- small mother against decapentaplegic 1 and 2; **FMOD** -
532 Fibromodulin.

533

Figure caption

Figure 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.

Figure 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 (*).

Figure 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.

Figure 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.

Figure 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

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 TGFβ1
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.
574
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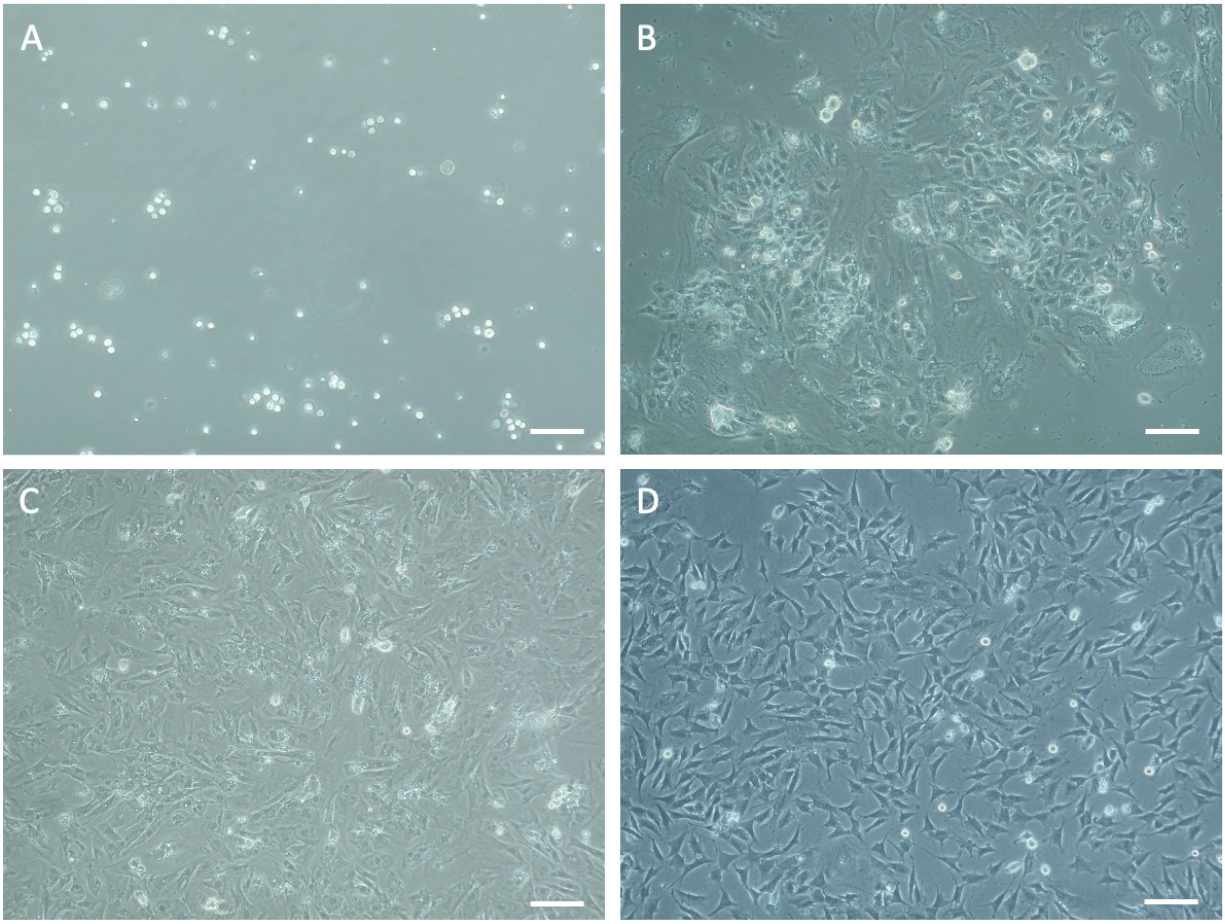

Fig. 1. Optical microscopy of freshly isolated chondrocytes after 1 day (A), 5 days (B) and 10 days (C) of primary culture; in fig. D a secondary culture.

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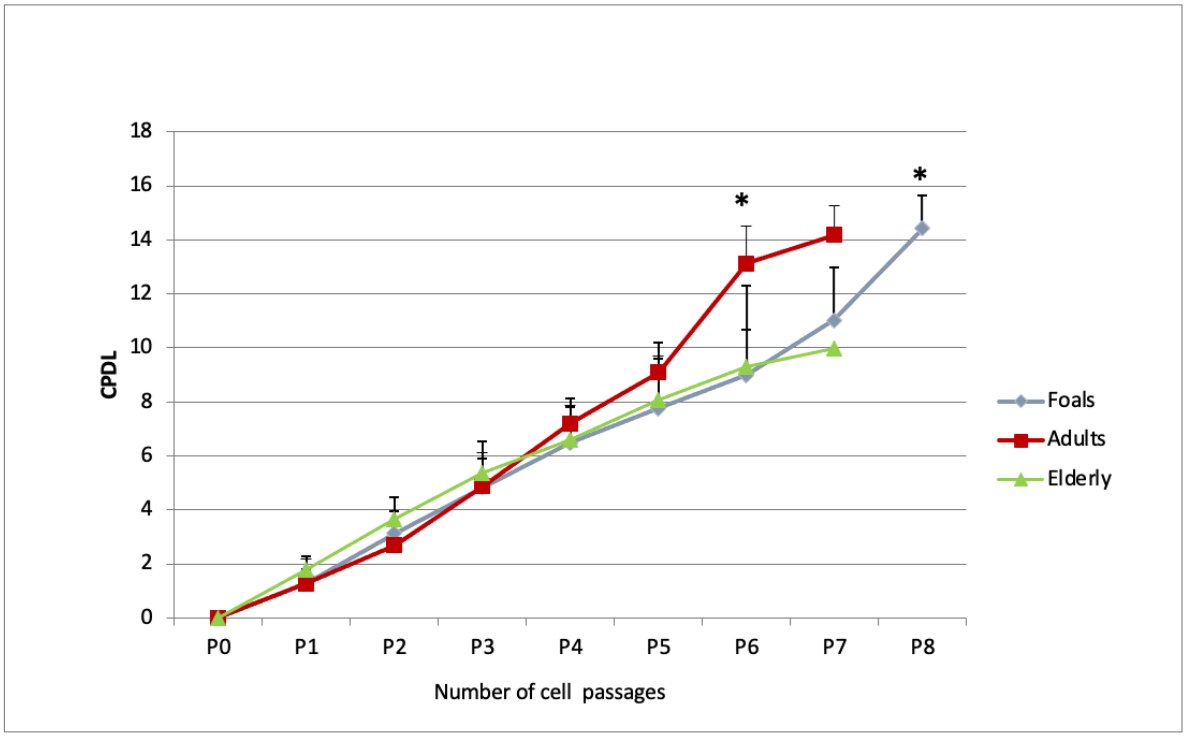

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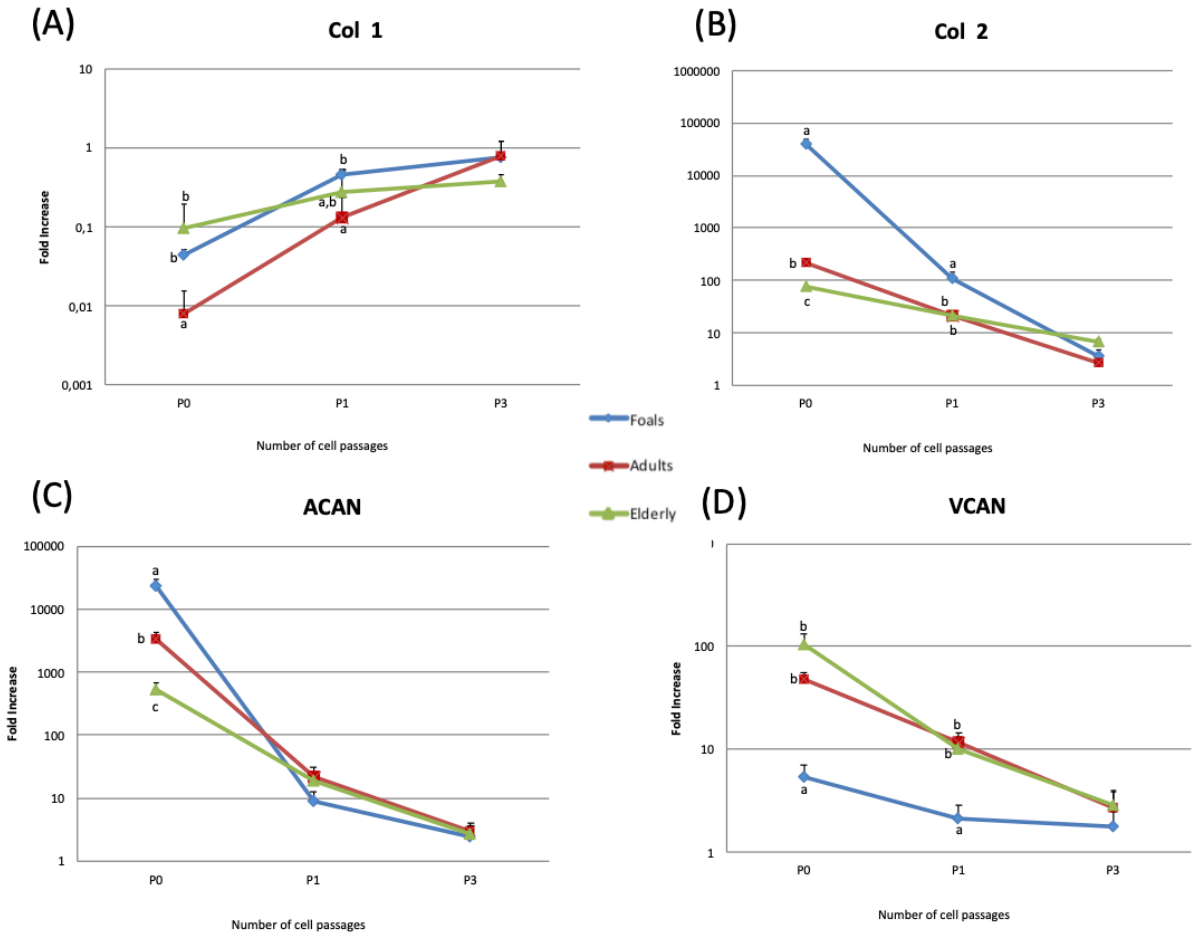

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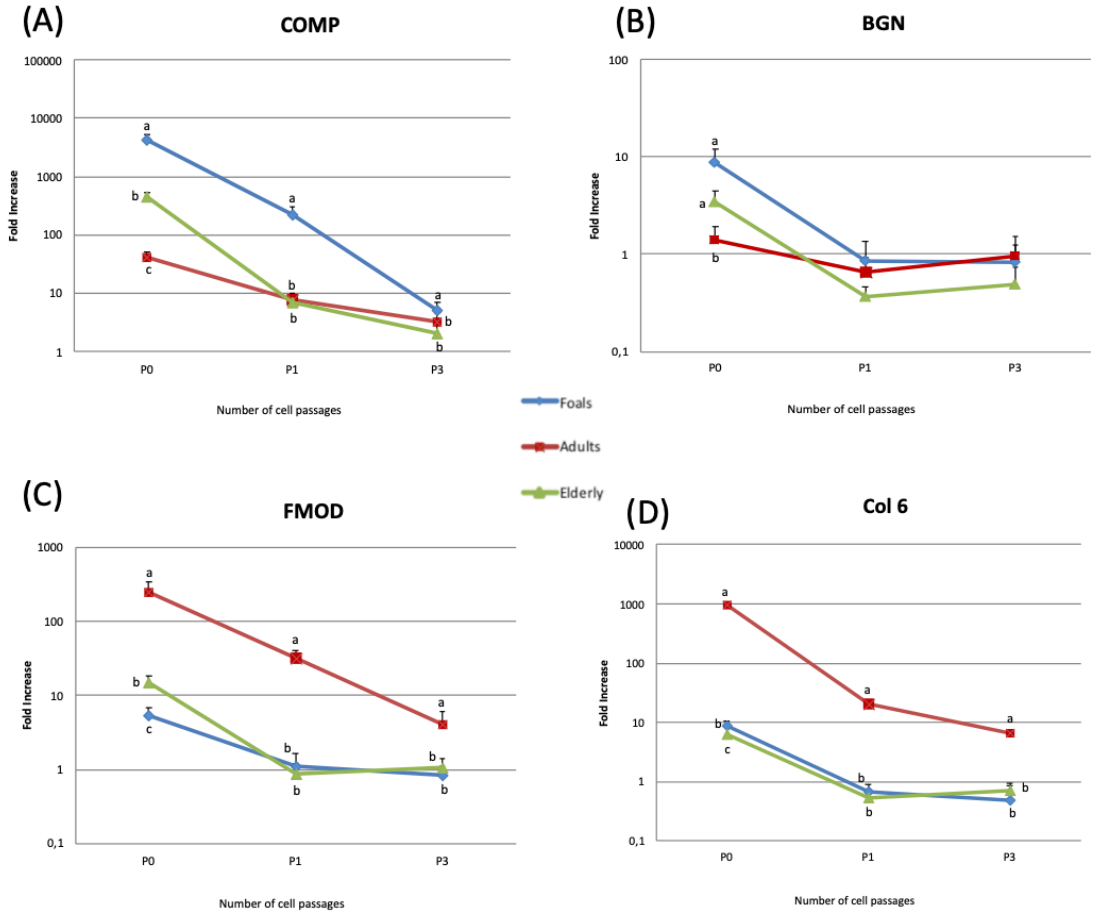

Fig. 4. Mean values of gene expression of Cartilage Oligomeric Matrix Protein (COMP) (A), Byglican (BGN) (B), Fibromodulin (FMOD) (C), Collagen Type VI (Col6) (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). 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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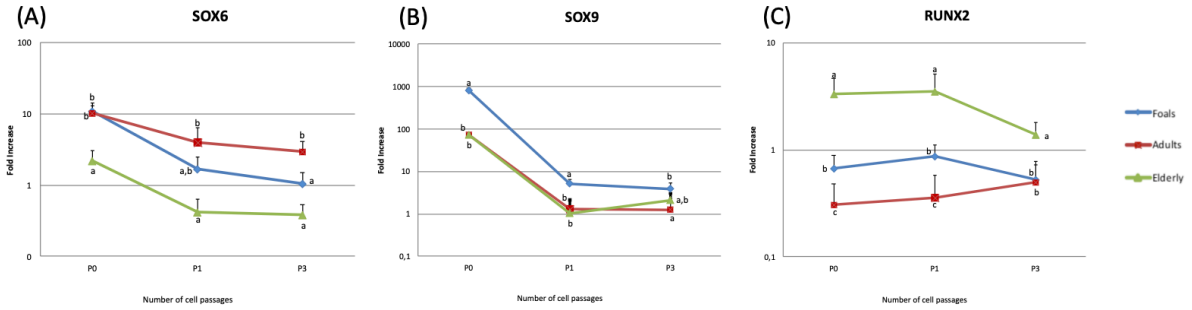

Fig. 5. Mean values of gene expression of SOX6 (A), SOX9 (B) and Runx2 (C) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences between groups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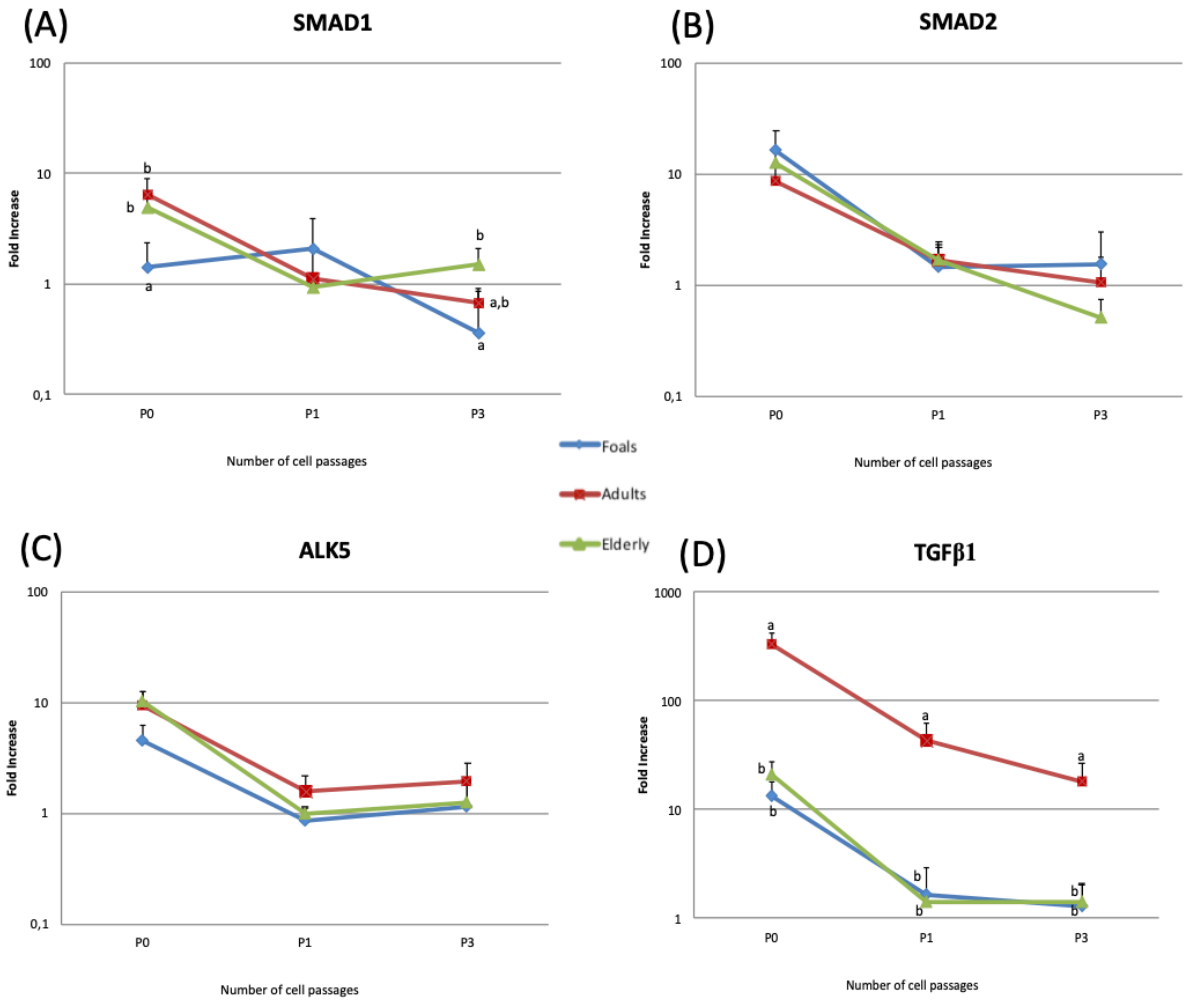

Fig. 6. Mean values of gene expression of and SMAD1 (A), SMAD2 (B), ALK5 (C) and TGFβ1 (D) in freshly isolated chondrocytes (P0) and subculturing (P1,P3) from animals of different age-dependent groups (foals, adult and elderly). Different superscript letters (a, b, c) refer to significant differences 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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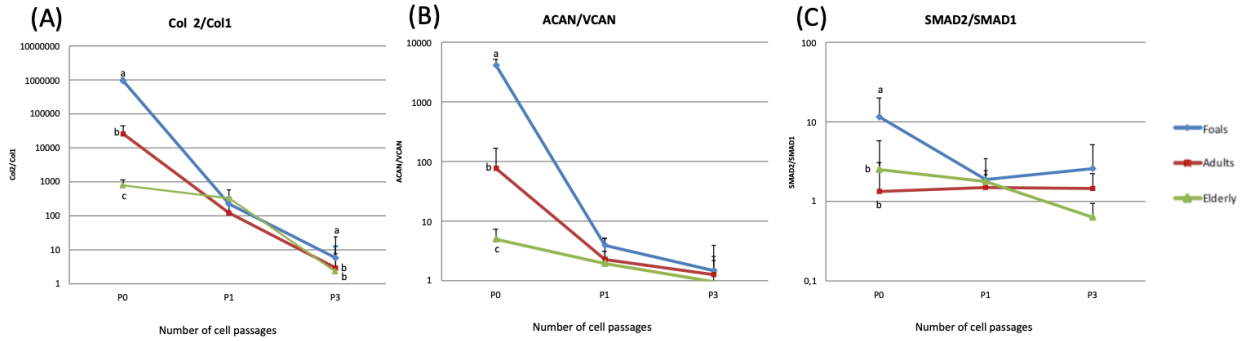

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