Austral J Vet Sci **49, 129-133 (2017)**

SHORT COMMUNICATION

Glycosaminoglycans (GAGs) determination in healthy and damaged equine articular cartilage

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ABSTRACT. The purpose of this study was to establish if there was any difference in the GAGs content between loaded and unloaded surfaces of the joint. Furthermore, the results were compared between macroscopically healthy and damaged joints. Cartilage samples were obtained from two different zones of the equine metacarpophalangeal joint (metacarpal condyles). Samples were collected from the loaded surface of macroscopically healthy joints (N1; n=10) and from macroscopically damaged cartilage (P1; n=10). Additionally, cartilage samples were collected from unloaded areas at the most dorso-proximal zone of the joint in macroscopically healthy joints (N2; n=10) and from the macroscopically pathological joints but without damaged cartilage on the site of sampling (P2; n=10). The GAGs were extracted from 100 mg of cartilage of each sample and quantified through the safranine - O method that measured the total anionic charges, and through the carbazole method that measured the uronic acid content. Both methods measured the GAGs content, showing no differences between intra-joint zones (1 and 2), but when the GAGs content was compared between healthy and pathological joints, both methods showed a significantly decreased GAGs content in the damaged joints (1 and 2). These results show that the whole articular cartilage could be affected in a chronic pathological process and is not only a local process occurring in the macroscopically damaged cartilage associated with the loaded area.

Key words: equine, joint, cartilage, glycosaminoglycans.

RESUMEN. El propósito de este estudio fue establecer la existencia de alguna diferencia en el contenido de GAG entre las superficies articulares que soportan y no soportan peso, comparando articulaciones metacarpofalángicas equinas macroscópicamente sanas y dañadas. Las muestras de cartílago se obtuvieron desde la superficie de apoyo de articulaciones macroscópicamente sanas (N1; n=10) y de aquellas macroscópicamente dañadas (P1; n=10). Adicionalmente, en una localización dorsoproximal se obtuvieron muestras de la superficie del cartílago sin apoyo, desde articulaciones sanas (N2; n=10) y dañadas (P2; n=10). Los GAG fueron extraídos desde 100 mg de cartílago de cada muestra y cuantificados mediante el método safranina-O que mide la carga aniónica total y por el método del carbazol que mide el contenido de ácido urónico. Ambos métodos fueron capaces de medir el contenido de GAG no encontrándose diferencias entre zonas intraarticulación (1 y 2). Al comparar el contenido de GAG entre articulaciones sanas y dañadas, ambos métodos evidenciaron una disminución significativa de GAG en las articulaciones dañadas (1 y 2). Estos resultados muestran que en un proceso patológico articular crónico se afecta el cartílago en su conjunto y no solo aquellas zonas que muestran lesiones macroscópicas al soportar mayor impacto mecánico.

Palabras claves: equino, articulación, cartílago, glicosaminoglicanos.

INTRODUCTION

Spontaneous joint disease is a common clinical problem in the horse. Surveys estimate that up to 60% of lameness are related to osteoarthritis (McIlwraith *et al* 2012) and they are generally related to high performance athletes. Usually, forelimbs are more affected than hindlimbs (Malikides 2007). The cartilage is a unique tissue lacking blood vessels and innervation, with 2 to 5% chondrocytes dispersed in the extracellular matrix (ECM) which mostly contains water (75%), collagen, mainly type II (15%) and proteoglycans (10%) (Malikides 2007). Type II collagen forms a network containing proteoglycans, which are high molecular weight molecules consisting of glycosaminoglycans (GAGs) covalently bound to a central protein. Aggrecan, also known as cartilage-specific proteoglycan, is the most abundant proteoglycan in the articular cartilage and its core protein is capable to bind up to 50 units of keratan sulfate and 100 units of chondroitin sulfate, being both very important GAGs in the ECM. Cartilage hyaluronan has the ability to bind aggrecan, forming large aggregated complexes (Palmer and Bertone 1994). All these molecules are critical components for cartilage structure and function of joints and they are responsible among others, for both the tensile and compression strength provided by type II collagen and proteoglycans, respectively (van der Harst *et al* 2005).

Degradation and loss of ECM components can be produced by apoptosis of chondrocytes, decreased metabolic activity through age possibly due to glycosylation processes (DeGroot *et al* 2001), and increased degradation by metalloproteinase (MMP) and aggrecanase activity during osteoarthritis. When the extracellular matrix is degraded, fragments of collagen and fibronectin are generated which stimulate the synthesis of proinflammatory cytokines, MMPs, and the immune response against the cartilage (Houard *et al* 2013).

Additionally, components of the extracellular matrix and cartilage thickness may increase due to dynamic compression (Guilak *et al* 2004), articular topography, and exercise intensity (Murray *et al* 2001, Tranquille *et al* 2009). However, *in vitro* studies have shown that excessive static compression causes proteoglycan loss

Accepted: 01.12.2016.

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and collagen network damage (Guilak *et al* 2004) with impaired cartilage function (Arokoski *et al* 2000, Goldring and Goldring 2007).

The purpose of this study was to quantify by two different methods, the concentration of GAGs extracted from two different locations within the cartilage of healthy and damaged metacarpophalangeal joints (metacarpal condyles). In pathological joints the samples were obtained from the loaded surface with macroscopically damaged cartilage and from the unloaded surface without macroscopically damaged cartilage. Both pathological areas were compared with their counterparts of the macroscopically healthy joints. We hypothesised that the amount of total GAGs concentration extracted from both areas of damaged cartilage would be less than in both areas of normal cartilage.

MATERIAL AND METHODS

SAMPLES

Cartilage samples were obtained *postmortem* from 2 to 8 years-old crossbreed horses with macroscopically normal metacarpophalangeal joints (n=10) and from horses between 5 - 12 years-old with macroscopically abnormal metacarpophalangeal joints (n=10). Samples were obtained right after slaughter. No previous physical examination was performed. Macroscopic appearance of joints was assessed through visual inspection right after arthrotomy. Age was determined approximately by dental chronometry. Each joint corresponded to one horse hence 20 horses were used.

Commonly, the loaded areas in the pathological joints showed signs of cartilage damage at visual inspection, situation not seen in the unloaded areas of pathological joints selected for this study. Normal joints showed a smooth and bright cartilage surface, pearly color, and a synovial membrane without signs of congestion. On the other hand, abnormal joints showed cartilage fibrillation, erosion and/or wear lines, changes in cartilage color, and synovial membrane congestion (Adarmes *et al* 2003).

Cartilage samples from the pathological joints were collected through a tangential cut using a scalpel blade from both medial and lateral condyles starting at the sagittal ridge all the way through the loaded surface of the joint (P1). Another set of samples was obtained starting 2 cm above P1 directed dorso-proximal (P2) representing the unloaded surface of the cartilage. Sampling for normal joints was conducted in the same manner as described for the pathological ones. Cartilage samples were kept in an ice bath and then frozen individually (-70ºC) until their processing.

GAGS EXTRACTION

Each 100 mg of cartilage were crushed and kept at 1:30 ratio with acetone for 24 hours at room temperature. Afterwards, samples were dried at 80ºC for 30 minutes and then digested with papain 0.1 mg/mL of phosphate buffer0.1M, EDTA 0.005 M, and cysteine-HCl 0.005 M pH 6.5. For digestion 1 mL enzymatic solution for every 20mg dried cartilage was used and kept for 48 hours at 65ºC.

Proteins were then precipitated with trichloroacetic acid 20% at 4°C for 12 hours. The precipitate was separated by centrifugation at 7,000 x g for 30 minutes at 4°C. The supernatant was dialysed with distilled water at 1:10 ratio for 48 hours at 4°C, and then every 24 hours for 3 times. The dialysate was treated with 4 volumes of sodium acetate 1% in 95% ethanol for 24 hours at room temperature allowing precipitation of GAGs. Glycosaminoglycans were separated by centrifugation at 7,000 x g for 30 minutes at 10°C. The pellet was dried at 40°C for 30 minutes, and then kept in a drying hood for 12 hours. The extracts obtained were weighed and stored at 4°C. Finally, an aqueous solution 0.2% was prepared from each sample in order to determine the total GAGs and uronic acids content (Nakano *et al* 1986).

TOTAL GAGS DETERMINATION

For GAGs determination, a spectrophotometric method was used. The technique is based on the use of Safranin O as cationic dye allowing quantification of GAGs anionic charges (Carrino *et al* 1991) (this technique includes the detection of chondroitin sulfate and keratan sulfate, the two GAGs that form constituting elements of the aggrecan). In brief, this method consisted of a dot blot system where a nitrocellulose membrane was loaded with 250 µL Safranin O 0.02% in sodium acetate 50 mM pH 4.8, and finally 25 µL of diluted sample 0.02% (5 µg GAGs) was added. Afterwards, vacuum was applied to remove the liquid fraction and then washed with sodium acetate 50 mM pH 4.8.The nitrocellulose membrane was then dissolved in 1 mL of cetilpiridinium 10% at 37°C for 20 minutes and absorbance was determined at 536 nm. 25 µL solution of chondroitin sulfate ranging between 2 to 8 µg was used as a standard.

URONIC ACID DETERMINATION

Chondroitin sulfate concentration can be determined indirectly measuring the uronic acid present in GAGs with the carbazole method (Bitter and Muir 1962). Briefly, GAGs solution 0.2% was diluted to 0.01%. Then, 2.5 mL of concentrated sulfuric acid containing tetraborate sodium 0.025 M were added to 500 µL of GAGs diluted solution (50 µg GAG). This mixture was kept in a water bath for 10 minutes, and then 250 µL carbazole 0.125% in absolute ethanol were added. The solution was kept in a water bath for 15 minutes for color development. The absorbance was determined at 530 nm. 500 µL sodium glucuronate ranging between 10 to 40 µg were used as a standard.

STATISTICAL ANALYSIS

Statistical analysis was run on InfoStat[1]. As some of the GAGs values were not normally distributed (Shapiro-Wilks test, $P<0.05$), non-parametric test were used. Wilcoxon test was applied for comparisons of data obtained from the same horse (N1-N2; P1-P2) while Mann-Whitney U test was performed for values coming from different horses (N1-P1; N2-P2). Significance threshold was set at $P<0.05$. Results were expressed as µg GAGs/mL ± SD.

RESULTS AND DISCUSSION

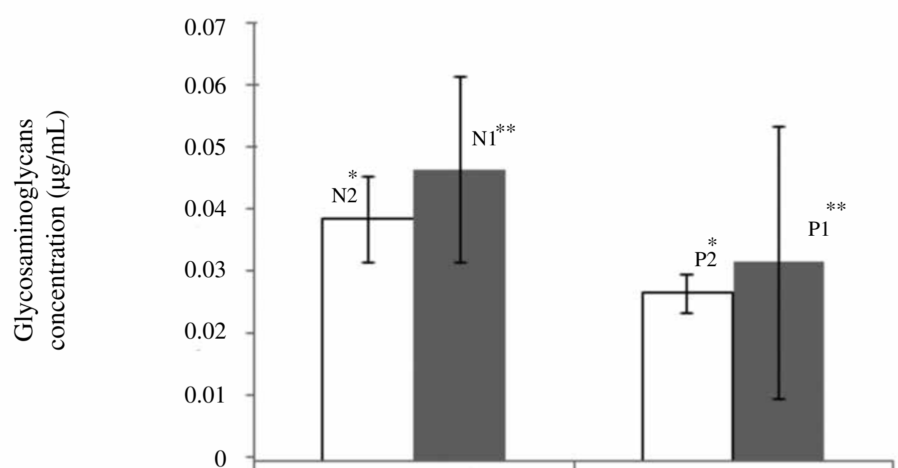
The GAG content (GAGs µg / mL) in normal joints determined by Safranin O test was 0.047 ± 0.015 (N1) and 0.039 ± 0.007 (N2) while in damaged joints it was 0.027 ± 0.003 (P1) and 0.032 ± 0.022 (P2) (figure 1). No statistically significant differences were found when both zones intra-joint were analysed but a significant decreased in GAGs concentration was seen in P1and P2 when compared with N1 and N2 ($P<0.05$).

Total GAG content (GAGs µg/mL) in normal joints determined by the carbazole method was 0.0050 ± 0.0009 (N1) and 0.0046 ± 0.0013 (N2) while in damaged joints was 0.0021± 0.0004 (P1) and 0.0022 ± 0.0011 (P2) (figure 2). No statistically significant differences were found when both zones intra-joint were analysed but a significant decrease in GAGs concentration was observed in macroscopically damaged joints (P1 and P2) when compared with normal joints (N1 and N2) ($P<0.05$).

Both tests used in this study were able to measure the GAGs concentration and both showed a significant decrease in GAGs content in damaged joints (P1 and P2). The Safranin O quantifies total GAGs by measuring anionic charges content present in chondroitin sulphate and keratan sulfate. Keratan sulfate contains one anionic charge while chondroitin sulphate contains two anionic charges. Chondroitin sulphate was used as standard due its quantitative importance in aggrecan and its content in anionic charges. Otherwise, the carbazole test measures the uronic content present in chondroitin sulphate but not in keratan sulfate, this situation could explain partially the lower concentration detected**,** approximately 11 fold less than with the Safranin O method, a more unspecific test.

The cartilage extracellular matrix decrease can be attributed to the damage produced by the action of pro-inflammatory mediators and the activation of MMPs together with cell death, such as necrosis and apoptosis, and decreased collagen and proteoglycan synthesis (Goldring and Goldring 2007, Houard *et al* 2013, Stevens *et al* 2009). In human knee osteoarthritis (OA) a decrease in chondroitin sulfate content was found as a result of chondroitin sulfate glycosyltransferase gene down-regulation (Ishimaru *et al* 2014). OA can be initiated by overload single-compression (Kurz *et al* 2005) as well as prolonged cyclic compression (Blain *et al* 2001) on articular cartilage. Equine articular

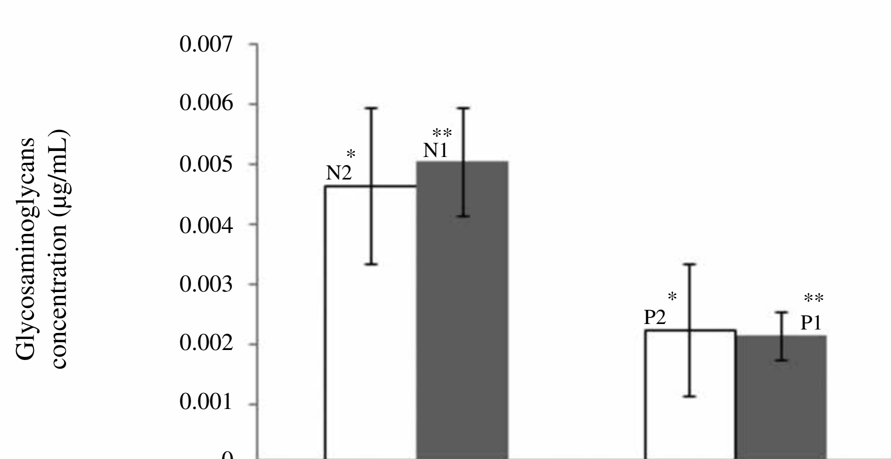
1 Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat versión 2014. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina; http://www.infostat.com.ar



	Healthy joint	Damaged joint
□ Non loaded surface	0.039±0.007	0.027±0.003
■ Loaded surface	0.047±0.015	0.032±0.022

Figure 1. Glycosaminoglycans concentration (µg/mL± SD) determined by Safranin O test and extracted from loaded and unloaded zones of healthy and damaged metacarpophalangeal joints.

**Significant difference between N1- P1 ($P<0.05$); * Significant difference between N2- P2 ($P<0.05$).



	Healthy joint	Damaged joint
□ Non loaded surface	0.0046±0.0013	0.0022±0.0011
■ Loaded surface	0.0050±0.0009	0.0021±0.0004

Figure 2. Glycosaminoglycans concentration (µg/mL± SD) determined by carbazole test and extracted from loaded and unloaded zones of healthy and damaged metacarpophalangeal joints.

**Significant difference between N1- P1 (P<0.05); *Significant difference between N2- P2 (P<0.05).

cartilage explants stimulated with interleukin-1β during 25 days released to culture media aggrecan fragments between days 3 and 6, similar to early-stage OA (Svala *et al* 2015). In early-stage OA, there is increased GAG synthesis and content in cartilage from areas flanking OA lesions compared to cartilage from macroscopically normal unaffected regions, while this content decreases in late-stage OA (Venkatesan *et al* 2012). The aforementioned could explain why there are no differences in cartilage thickness between normal and moderately damaged joints, despite the differences in the biomechanical properties of two different areas within the cartilage along with degenerative changes in the first phalanx of the metacarpophalangeal joint (Brommer *et al* 2005). Therefore, the results of this study would be more likely related to the selection of markedly damaged joints like late-stage OA instead of moderately damaged joints.

Both groups of horses selected for this study had a wide range of age: 2 to 8 years-old for normal joints and 5 to 12 years-old for abnormal joints. It has been reported that the total GAGs content of the metacarpophalangeal joint remains relatively constant through age, and it has been described that changes only occur in the sulfation pattern of chondroitin sulphate (Platt *et al* 1998). On the other hand, Hui *et al* (2016) described a progressive loss of extracellular matrix and cellularity related with aging, nevertheless, that study was conducted in mice ranging between 3 and 30 months old. Thus, we suggest that age range of the animals selected did not affect the results obtained in this study.

Exercise has been related with an increase in cartilage thickness (Tranquille *et al* 2009) and proteoglycan content, especially newly formed small monomers (Palmer *et al* 1995). Palmer *et al* (1995) took cartilage samples from different places within the third carpal bone and no differences were seen, regarding the content of newly formed proteoglycans. The same study showed that total endogenous or pre-existing proteoglycan content did not change with exercise, but when they compared the proteoglycan content between different sites within the joint, significant differences were found, which could be related to biomechanical factors (Palmer *et al* 1995, Brommer *et al* 2005). In humans, it has been stated that both the thickness and cartilage composition, depend on mechanical and biomechanical factors including muscle contraction (Ganse *et al* 2015). The crossbreed horses selected for our study, did not usually have a sporting purpose and therefore this variable probably did not influence the results. Additionally, the study excluded Thoroughbred horses associated with competitive exercise.

On the other hand, each normal or damaged joint showed no difference between both areas of cartilage analysed, although the middle area of the condyles supports greater mechanical load and would be more likely to develop lesions (Harrison *et al* 2014) with a decrease in the content of GAGs. The interesting thing about these results is that in badly damaged joints the cartilage is affected as a whole, it would be subjected to conditions that reduce the content of GAGs and not just those areas that support greater mechanical stress, which will ultimately affect functionality.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Rigoberto Solís for his assistance with statistics, Gabriel Manríquez for his assistance with image analysis and Víctor Molina for his assistance during the study.

REFERENCES

Adarmes H, Riveros A, Galleguillos M, González E. 2003. Contenido de glicosaminoglicanos del líquido sinovial de la articulación metacarpofalángica de caballos castrados y yeguas de diferentes edades. *Arch Med Vet* 35, 51-59.

Arokoski JPA, Jurvelin JS, Väätäinen U, Helminen HJ. 2000. Normal and pathological adaptations of articular cartilage to joint loading. *Scand J Med Sci Sports* 10, 186-198.

Bitter T, Muir HM. 1962. A modified uronic acid carbazole reaction. *Ann Biochem* 4, 330-334.

Blain E, Gilbert S, Wardale R, Capper S, Mason D, *et al*. 2001. Up-regulation of matrix metalloproteinase expression and activation following cyclical compressive loading of articular cartilage *in vitro*. *Arch Biochem Biophys* 396, 49-55.

Brommer H, Laasanen MS, Brama PAJ, van Weeren PR, Helminen HJ, *et al*. 2005. Functional consequences of cartilage degeneration in the equine metacarpophalangeal joint: quantitative assessment of cartilage stiffness. *Equine Vet J* 37, 462-467.

Carrino DA, Arias JL, Caplan AI. 1991. A spectrophotometric modification of a sensitive densitometric safranin O assay for glycosaminoglycans. *Biochem Inter* 24,485-495.

DeGroot J, Verzijl N, Jacobs KMG, Budde M, Bank RA, *et al*. 2001. Accumulation of advanced glycation endproducts reduces chondrocytes-mediated extracellular matrix turnover in human articular cartilage. *Osteoarthr Cartilage* 9, 720-726.

Ganse B, Zange J, Weber T, Pohle-Fröhlich R, Johannes BW, *et al*. 2015. Muscular forces affect the glycosaminoglycan content of joint cartilage. *Acta Orthopaedica* 86, 388-392.

Goldring MB, Goldring SR. 2007. Osteoarthritis. *J Cell Physiol* 213, 626-634.

Guilak F, Fermor B, Keefe FJ, Kraus VB, Olson SA, *et al*. 2004. The role of biomechanics and imflammation in cartilage injury and repair. *Clin Orthop Relat Res* 427, 17-26.

Harrison SM, Whitton RC, Kawcak CE, Stover SM. 2014. Evaluation of subject-specific finite-element model of the equine metacarpophalangeal joint under physiological load. *J Biomech* 47, 65-73.

Houard X, Goldring MB, Berenbaum F. 2013. Homeostatic mechanism in articular cartilage and role of inflammation in osteoarthritis. *Curr Rheumatol Rep* 15, 375-384.

Hui W, Young DA, Rowan AD, Xu X, Cawston TE, *et al*. 2016. Oxidative changes and signaling pathways are pivotal in initiating age-related changes in articular cartilage. *Ann Rheum Dis* 75, 449-458.

Ishimaru D, Sugiura N, Akiyama H, Watanabe H, Matsumoto K. 2014. Alterations in the chondroitin sulfate chain in human osteoarthritic cartilage of the knee. *Osteoarthr Cartilage* 22, 250-258.

Kurz B, Lemke AK, Fay J, Pufe T, Grodzinsky AJ, *et al*. 2005. Pathomechanisms of cartilage destruction by mechanical injury. *Ann Anat* 187, 473-485.

Malikides N. 2007. Equine lameness. In: McGowan CM, Stubbs N, Goff L (eds). *Animal physiotherapy: assessment, treatment and rehabilitation of animals.* Blackwell, Oxford, UK, Pp 82.

McIlwraith CW, Frisbie DD, Kawcak CE. 2012. The horse as a model of naturally occurring osteoarthritis. *Bone Joint Res* 1, 297-309.

Murray RC, Birch HL, Lakhani K, Goodship AE. 2001. Biochemical composition of equine carpal articular cartilage is influenced by short-term exercise in a site-specific manner. *Osteoarthr Cartilage* 9, 625-632.

Nakano T, Thompson JR, Aherne FX. 1986. Distribution of glycosaminoglycans and the nonreducible collagen crosslink pyridinoline in porcine menisci. *Can Vet Res* 50, 532-536.

Palmer JL, Bertone AL. 1994. Joint structure, biochemistry and biochemical disequilibrium in synovitis and equine joint disease. *Equine Vet J* 26, 263-277.

Palmer JL, Bertone AL, Malemud CJ, Carter BG, Papay RS, *et al*. 1995. Site-specific proteoglycan characteristics of third carpal articular cartilage in exercised and nonexercised horses. *Am J Vet Res* 56, 1570-1576.

Platt D, Bird JLE, Bayliss MT. 1998. Ageing of equine articular cartilage: structure and composition of aggrecan and decorin. *Equine Vet J* 30, 43-52.

Stevens AL, Wishnok JS, White FM, Grodzinsky AJ, Tannenbaum SR. 2009. Mechanical injury and cytokines cause loss of cartilage integrity and upregulate proteins associated with catabolism, immunity, inflammation, and repair. *Mol Cell Proteomics* 8, 1475-1489.

Svala E, Löfgren M, Sihlbom C, Rüetschi U, Lindahl A, *et al*. 2015. An inflammatory equine model demonstrates dynamic changes of inmune response and cartilage matrix molecules degradation *in vitro*. *Connect Tissue Res* 56, 315-325.

Tranquille CA, Blunden A, Dyson SJ, Parkin TDH, Goodship AE, *et al*. 2009. Effect of exercise on thicknesses of mature hyaline cartilage, calcified cartilage and subchondral bone of equine tarsi. *Am J Vet Res* 70, 1477-1483.

Van der Harst MR, van de Lest CHA, Degroot J, Kiers GH, Brama PAJ, *et al*. 2005. Study of cartilage and bone layers of the bearing surface of the equine metacarpophalangeal joint relative to different timescales of maturation. *Equine Vet J* 37, 200-206.

Venkatesan N, Barré L, Bourhim M, Magdalou J, Mainard D, *et al*. 2012. Xylosiltrasferase-I regulates glycosaminoglycan synthesis during the pathogenic process of human osteoarthritis. *PLoS ONE* 7, e34020. doi:10.1371/journal.pone.0034020.