A case study: Glycosaminoglycan profiles of autologous chondrocyte implantation (ACI) tissue improve as the tissue matures.

Aarti Sharma, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA.

Email: as3182@cumc.columbia.edu

Dai Rees, Sports Injury Services, Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG, UK.

Sally Roberts, Institute of Science & Technology in Medicine, University of Keele, Arthritis Research Centre, Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG, UK.

Email: sally.roberts@rjah.nhs.uk

Nicola J Kuiper, Institute of Science & Technology in Medicine, University of Keele, Arthritis Research Centre, Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG, UK.

Email: n.j.kuiper@keele.ac.uk

Corresponding author: Nicola J Kuiper

Background Autologous chondrocyte implantation (ACI) has been used to treat cartilage defects in thousands of patients worldwide with good clinical effectiveness 10-20 years after implantation. Information concerning the quality of the repair cartilage is still limited because biopsies are small and rare. Glycosaminoglycan structure influences physiological function and is likely to be important in the long term stability of the repair tissue. The aim of this study was to assess glycosaminoglycans in ACI tissue over a 2 year period.

Methods Biopsies were taken from one patient (25 years old) at 12 months and 20 months post ACI-treatment and from three normal cadavers (21, 22 and 25 years old). Fluorophore-assisted carbohydrate electrophoresis (FACE) was used to quantitatively assess the individual glycosaminoglycans.

Results At 12 months the ACI biopsy had 40% less hyaluronan than the age-matched cadaveric biopsies but by 20 months the ACI biopsy had the same amount of hyaluronan as the controls. Both the 12 and 20 month ACI biopsies had less chondroitin sulphate disaccharides and shorter chondroitin sulphate chains than the age-matched cadaveric biopsies. However, chondroitin sulphate chain length doubled as the ACI repair tissue matured at 12 months (3913Da±464) and 20 months (6923Da±711) and there was less keratan sulphate as compared to the controls.

Conclusions Although the glycosaminoglycan composition of the repair tissue is not identical to mature articular cartilage its quality continues to improve with time.

Keywords autologous chondrocyte implantation, glycosaminoglycans, cartilage, Fluorophore assisted carbohydrate electrophoresis (FACE), hyaluronan

Introduction

Autologous chondrocyte implantation (ACI) was first introduced as a biological cell therapy approach to treat small focal defects of articular cartilage[1]. To date, ACI has been used to treat cartilage defects in thousands of patients worldwide with good clinical effectiveness several years after implantation[2,3]. Many centres report a high percentage of good to excellent clinical results with a failure rate of ~15%[4,5]. The majority of failures occur within the first year. Only a small number of centres, including our own, have investigated the long-term stability of the repair tissue using outcome measures such as mechanical testing, clinical scores, MRI and histology[6-8].

Previous studies of patient knee function scores following ACI have indicated the importance of a two-year post-operative status as an indication of the long term success of this treatment[2,9,10]. One study, which tracked patients for two to nine years post ACI[2], showed that the percentage of good to excellent results was 92% in patients with isolated femoral condyle lesions. Another study found improved joint function and a better quality of life in 72% of patients 12 months post ACI and improved joint function in 84% of patients 3 years post operatively[9]. These results are encouraging but there are still concerns about the quality of the cartilage formed at the site of the defect. Detailed assessment of the repair tissue has been restricted by the size and availability of ACI biopsies. Basic histological studies of ACI biopsies indicate that repair tissue matures slowly[11-13]. Further detailed studies are necessary to fully assess the molecular characteristics of the tissue as it matures over time.

The unique properties of articular cartilage depend on the interactions between the chondrocytes with their extracellular matrix[14]. Glycosaminoglycans (GAGs) surround the chondrocyte linking them to other chondrocytes and to the rest of the extracellular matrix. Chondroitin sulphate (CS), keratan sulphate (KS) and hyaluronan (HA) are GAGs found in articular cartilage where they provide both physiological and biological functions[15]. Each GAG has a distinctive molecular composition, which typically comprises a repeating disaccharide unit of an amino sugar and a uronic acid. GAGs acquire a considerable degree of complexity through their extensive modifications which involve sulphation and epimerisation. Due to these complexities, it has always been difficult to demonstrate clear structure-function relationships of GAGs. Studies, including our own, have shown that GAGs in articular cartilage change as a function of development, age and disease[16-23]. In adult cartilage, 50-80% of GAGs are CS chains which are composed of glucuronic acid and N-acetylgalactosamine monosaccharides. N-acetylgalactosamine monosaccharides are sulphated on carbon positions 4 (C4S) and/or 6 (C6S), and the CS chain is terminated with a N-acetylgalactosamine sugar that can be sulphated[16]. During development, chondroitin sulphation is thought to be tightly controlled both spatially and temporally[17]. During embryogenesis CS chains are exclusively C6S. From foetal development to adolescence, CS chains tend to be equally C4S and C6S. In this age range, CS chains are ~25kD and almost entirely terminated with a 4-sulphated N-acetylgalactosamine[18]. During adulthood, CS chains tend to have more C6S than C4S, the chains are shorter at ~16kD, and they are terminated with a 4- or 6-sulphated N-acetylgalactosamine[8,22], or a 4,6-

sulphated N-acetylgalactosamine[18]. KS represents 20-50% of the GAG chains in adult cartilage. KS chains are more complex than CS chains because they can have a branched structure. This makes it difficult to determine their chain length and termination sugars. KS is composed of galactose and N-acetylglucosamine. KS chains can be modified by O-sulphation of the hydroxyl groups, but in a much more restricted pattern when compared with CS. O-sulphation of the hydroxyl groups at C6 of both the galactose and/or the N-acetylglucosamine, results in un-, mono- or disulphated repeat disaccharides. The sulphation levels of KS chains isolated from young cartilage (5-11 yr-old) have been shown to increase from 30-50% to 80-95% with advancing age[19]. KS chains also demonstrate an age-related increase in chain length[20]. HA has been reported to account for 1-10% of GAGs in articular cartilage although higher values have been reported[8,21-23]. HA is composed of unsulphated glucuronic acid and unsulphated N-acetylglucosamine monosaccharides. The exact composition of the terminal sugar is currently unknown. High concentrations of HA are generally found during aging and development.

To date, the GAG properties in ACI repair tissue are largely unknown. Defining the molecular composition of GAGs in repair cartilage over time may help us to understand how the tissue matures. Immunological assessment of ACI biopsies[20,24] have defined the distribution of GAGs but these approaches are limited in characterising sulphation pattern or the real quantitation of GAGs. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) has evolved over the last decade to be a highly sensitive technique for profiling GAGs[8,22,25-27]. Our lab has used FACE in a range of applications

including describing age-related changes in GAGs in a limited number of ACI biopsies[8] and through the zones of normal, healthy cartilage[22]. Herein we have used FACE to quantify the specific GAGs in ACI biopsies removed at 12 and 20 months post-surgery in order to provide some insight into the changes in GAGs within maturing tissue.

Materials and Methods.

Autologous chondrocyte implantation (ACI) biopsies: A 25 year old with an isolated femoral condyle lesion underwent ACI and had a biopsy (1.8 mm in diameter, ~5 mm in length) removed during a follow up arthroscopy at 12 months. The biopsy was taken perpendicularly from the articulating surface through the full depth of cartilage and subchondral bone. A further biopsy was removed during a second follow up arthroscopy at 20 months. The removal of the two biopsies was given ethical approval by the Shropshire Research and Ethics Committee (UK) and the patient was consented at the time of each surgery.

Cadaveric tissue: An age-range (21, 22 and 25 years) of cadaveric knees was obtained from the UK Human Tissue Bank with full approval by Trent Research Ethics Committee. All of the cadavers had been involved in road traffic accidents. Prosected knees were obtained within 24h of death. All articular cartilage appeared healthy and macroscopically normal.

Histology: Core biopsies of the cadaveric and repair tissue formed were obtained and processed as previously described[8]. These were snap-frozen in liquid nitrogen–cooled hexane prior to sectioning and 7-μm thick cryosections were collected onto poly-L-lysine–coated slides. Sections were stained with toluidine blue and viewed with bright light microscopy to assess the glycosaminoglycan content of the tissue and polarised light to show the arrangement of the collagen fibrils.

Glycosaminoglycan (GAG) analysis: GAGs were extracted using an adaptation as previously described[8,22]. Briefly, the biopsies (full depth of cartilage, ~250 μg) were digested at 60°C with 125 μg proteinase K in 100 μl

of 100 mM ammonium acetate buffer, pH 7.0 for 4 h. GAGs were ethanol precipitated (final concentration of 80% v/v) from the proteinase K digests and aliquots of each biopsy were further enzymatically processed. All GAG-specific enzymes and standards were purchased from Seikagaku. HA was digested with 100 mU/ml of hyaluronidase SD (Hyase SD) for 1 h at 37 °C. CS was digested with 100 mU/ml of chondroitinase ABC (cABC) for 3 h at 37°C. The sulphation pattern of CS was confirmed by incubation of cABC-digested samples with 100 mU/ml of chondroitin-4ase and/or chondroitin-6ase for 12 h at 37 °C. Non-reducing termini of CS were identified by mercuric ion treatment. For KS analysis, samples were incubated with 100 mU/ml of keratanase II for 3h at 37 °C or 100 mU/ml of endo-β-galactosidase for 14 h. KS digestion was confirmed by sequential digestion with 100 mU/ml of keratanase II for 3 h and 100 mU/ml of endo-β-galactosidase for 14 h and conversely with 100 mU/ml of endo-β-galactosidase for 14 h and 100 mU/ml of keratanase II for 3 h.

Lyophilised enzyme-digested samples and standards were fluorescently labelled for 16 h at 37 °C for 16 h with 5 μl 12.5 mM 2-aminoacridone and 5 μl of 1.25 M sodium cyanoborohydride. Following fluorescent tagging, 10 μl of 25% glycerol (20% v/v final concentration) was used to quench excess sodium cyanoborohydride. Portions (5μl) of these samples were electrophoretically separated using a T25 %/C3.75 % acrylamide resolving gel in 187.5 mM Tris-borate and 187.5 mM Tris-HCl buffer and T5 %/C1.5 % acrylamide stacking gel in 360 mM Tris-HCl buffer.

Gels were placed on a transilluminator light box fitted with a 312-nm light source. Fluorescent images were captured using a High CCD Camera (UVP,

Cambridge, UK) and the mean pixel density for each product band was quantified using LAB WORKS Software (UVP). For each gel, FACE product bands were identified by their co-electrophoresis with a range of pre-defined fluorotagged saccharide standards as previously described[8,22]. Accurate quantitation was achieved between 10 and 400 pmol of product.

Statistical analysis: Each biopsy was analysed 8 times and results are represented as the mean ± standard error of the mean (SEM). All analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, California).

Results

Histology: Representative toluidine blue stained sections from knee cartilage obtained from a 22 year old cadaver and the 25 year old patient at 12 months and 20 months post-ACI are shown in Figure 1. Tissue obtained from the 22 year old cadaver (A) shows good homogenous metachromasia and good integration with the underlying subchondral bone. For the patient at 12 months post-ACI (B), metachromasia is more intense nearer to the subchondral bone and there appears to be reasonable integration with the bone. Less metachromasia towards the top of the tissue could represent the remnants of the periosteal patch. Polarising light (C) shows random arrangement of collagen fibrils within the tissue at low and high power (insert). This is in agreement with our previously published work which showed that at 12 months most biopsy tissue was either fibrocartilaginous or mixed morphology[24,28,29]. For the patient at 22 months post-ACI (D), there is clearly more metachromasia throughout the tissue than at 12 months post-ACI. The intact surface of the cartilage has the strongest staining. There is also good integration with the underlying bone. Neither of the post-ACI biopsies are as strongly stained as the cadaveric tissue which suggests that there is less GAG post-ACI. For the patient at 22 months post-ACI (E), polarising light still shows a random arrangement of collagen fibrils within the tissue but at high power (insert) there are clearly more closely packed collagen fibrils compared with the 12 month post-ACI biopsy.

Proportion of CS, HA and KS in the biopsies: FACE analysis revealed the differences in the proportions of HA, CS and KS between ACI biopsies and age-matched cadaveric biopsies (Figure 2). Each GAG, with its constituent

saccharide component, was expressed as a total percentage of GAGs analysed. There was more CS in both of the ACI biopsies than there was in the age-matched controls. Conversely, there was less KS in both of the ACI biopsies in comparison with the age-matched controls. In the 12 month ACI biopsy, HA accounted for 13.72±0.39% of the total GAG (Figure 2). This was much lower than the 20 month ACI biopsy and the cadaver controls (Figure 2, 6.46±0.26% and 6.09±1.31%).

FACE analysis of chondroitinase and hyaluronidase digestion products: Figure 3A shows the concentration of CS (ΔDiOS, ΔDi6S and ΔDi4S) and HA disaccharides in the biopsies. FACE revealed that the concentration of CS disaccharides was consistent within the age-matched controls; ΔDi6S (1.28±0.17μg/mg, 62%), ΔDi4S (0.56±0.09μg/mg, 27%) and ΔDi0S (0.24±0.08μg/mg, 11%). The 12 month and the 20 month ACI biopsies had less CS disaccharides than the age-matched controls (Figure 3A). The CS disaccharide compositions for 12 month and 20 month ACI biopsies were ΔDi6S (32%), ΔDi4S (37%), and ΔDi0S (31%); and ΔDi6S (47%), ΔDi4S (20%), and ΔDi0S (33%), respectively.

At 12 months the ACI biopsy had 0.24μg/mg HA (Figure 3A) but as the ACI repair tissue matured the HA concentration increased to the same level as the controls. At 20 months the ACI biopsy had 0.37±0.1μg/mg and the cadaveric biopsies had 0.36±0.09μg/mg. These data compare well with previously published data for adult articular cartilage[8,17,22].

Analysis of the terminal sugars of CS: Adult CS chains are predominantly terminated with GalNAc4S, GalNAc6S or GalNAc4,6S. For both the ACI repair tissue and control tissues, analysis of the terminal sugars of CS

identified low levels of GalNAc and GalNAc6S but no GalNAc4,6S (Figure 3B). Previous studies, including our own, have not detected GalNAc4,6S[8,17,22]. The concentration of terminal sugars increased as the repair tissue matured.

FACE analysis of keratanase II and endo-β-galactosidase digestion products: Within the ACI repair tissue there was a lower concentration of KS compared the cadaveric tissue (Figure 3C). We did not detect any unsulphated disaccharides in either the ACI or cadaveric biopsies which is consistent with the literature[19,20,22]. The profile within the cadaveric tissue was similar to our previously published data[8,22], in that a range of sulphated disaccharides and a terminal capping residue were detected. In the control biopsies we detected two monosulphated disaccharides (Gal-GlcNAc6S, GlcNAc6S-Gal) and a disulphated disaccharide (Gal6S-GlcNAc6S) from the KS chain interior, and a non-reducing terminal sialic acid trisaccharide (Neu-Gal-GlcNAc6S). Gal6S-GlcNAc6S was present in the highest concentration in all controls (1.51±0.06μg/mg) and it doubled in concentration as the ACI tissue matured (12 months 0.14±0.01μg/mg, 20 months 0.29±0.07μg/mg).

The ratio of GAGs: During cartilage maturation, CS undergoes major changes in sulphation up to the age of 20 years; ΔDi6S content increases whilst ΔDi4S content decreases. All three cadaveric biopsies had more ΔDi6S than ΔDi4S and this was reflected in the ratios of ΔDi6S:ΔDi4S (Figure 4A). At 12 months post ACI, the ratio of ΔDi6S:Di4S was significantly lower than the control data. At 20 month post ACI, the ratio increased and approached the levels of the age-matched controls. The ratio of CS:KS was significantly lower in all controls than in the ACI biopsies (Figure 4B).

Determination of the average CS chain size: The value for the average CS chain length was determined by dividing the total fluorescence of the internal disaccharides by the total fluorescence of the non-reducing termini as previously described[8,25]. In the control group the chain length was 18.21±0.16 kDa (Figure 4C). By contrast, CS chains in the repair tissue were significantly shorter (12 months 3.91±0.46 kDa, 20 months 6.92±0.71 kDa). The CS chain length doubled as the ACI repair tissue matured but this increase was not significant.

Discussion.

Previous studies of patient knee function scores following ACI have indicated the importance of a two-year post-operative status as an indicator of the long term success of this treatment[2,3,9,10]. Whilst these results are reassuring there remains concern about the quality of the cartilage formed within the defect. For cartilage repair tissue, there have been many studies assessing the collagen[28-30] but a very limited number of studies assessing proteoglycans and GAGs. Proteoglycans, with their associated highly anionic GAGs, are key players in a range of cartilage functions including tissue compressibility, tissue remodelling, up-take of proteins, intracellular signalling and cell movement[31]. In our previous work[22], applying FACE to analyse normal, healthy articular cartilage, we have shown that the middle zone has a higher concentration of HA than the superficial and deep zones. HA interacts with aggrecan to retain water in cartilage giving the tissue its compressibility. Thus the HA-rich middle zone might be very important in influencing the swelling behaviour of the cartilage, and in turn the associated compressibility. We demonstrated an increased ΔDi6S to ΔDi4S in the deep and middle zones compared to the superficial zones[22] and we noted that the sulphation levels of KS increased from around 50-80% with advancing age[22]. All of this information is providing us with clues to further understand the importance of cartilage GAGs.

Previously, also utilising FACE, we have shown that 12 month ACI repair tissue contains less CS and KS but more HA when compared to age-matched control cartilage[8]. Furthermore, CS chains were significantly shorter in 12 month ACI repair tissue[8]. Many studies have reported on maturation of chondrocytes in pellets[31] and cartilage like tissue in scaffolds[32] but very few

studies have reported on tissue maturation within a cartilage defect[11,12,29]. From our limited knowledge, we do know that the maturation of the tissue as a whole is very slow. Therefore in this study we have carried out a detailed analysis of the GAGs to assess how they behave in the repair tissue as it matures over time. Clearly the limitation of our study is that we have only presented the results from a single patient. This is because the ACI biopsies are rare. Despite this, our work has given us a preliminary handle on the difference between the chemistries and concentrations of GAGs in the ACI repair tissue. ACI biopsies removed at 12 and 20 months contained fewer GAGs than the age-matched controls. HA and CS levels increased as the repair tissue matured but KS levels remained low.

Reduced GAG levels in ACI repair tissue could simply be due to the expansion of the chondrocytes prior to implantation. Articular chondrocytes *in vivo* synthesise and secrete an extracellular matrix that is rich in collagens, proteoglycans, GAGs and non-collagenous proteins. Freshly isolated articular chondrocytes that are cultured in monolayer are known to undergo de-differentiation[34] and subsequently produce different proteoglycans[35]. After extensive monolayer culture, de-differentiated chondrocytes will irreversibly lose their chondrogenic potential[35]. At our centre, freshly isolated chondrocytes for ACI are cultured in monolayer for a short period of time to avoid the loss of their chondrogenic potential[36]. The effect of *in vitro* expansion on implanted cells has yet to be investigated in full. Liu *et al.* measured the amount of newly synthesised GAGs and the patterns of sulphation by serially subcultured monolayer articular chondrocytes following their re-differentiation in 3-dimensional culture[37]. The ∆Di6S:Di4S ratio of the

articular chondrocytes decreased when the cells were cultured in monolayer and then increased dramatically when the cells were placed in 3-dimensional culture[37]. The production of proteoglycans and their associated GAGs returned to near normal levels but only after several weeks in 3-dimensional culture. Liu *et al*. demonstrated that the reversal of gene expression of articular chondrocytes after monolayer culture and then re-differentiation is time dependent. In our study, the cells for ACI clearly need a long period *in vivo* to regain their phenotype but we have no direct comparison to estimate just how long this recovery takes.

Even though ACI remains a successful technique especially in articular cartilage restoration, there is on-going research to improve the technique[5]. A modification of the ACI technique has been achieved with matrix-induced autologous chondrocyte implantation (MACI), in which autologous chondrocytes are seeded and cultured on collagen membranes before implantation. This approach provides the cells with a 3-dimensional environment prior to implantation which is lacking in the original ACI technique. It is widely accepted that cartilage remodelling following chondrocyte expansion is notoriously slow[21,38]. Freshly synthesised proteoglycans do not form aggregates and quickly diffuses out of the cartilage[38]. Thus the lack of a 3-dimensional environment and the lack of ability to form aggregates may result in the GAGs and their associated proteoglycans being lost from the tissue for a period of time after cell implantation. Furthermore, re-differentiated chondrocytes in a 3 dimensional environment will synthesise less GAGs if they are too closely packed together or even too far apart[39]. Taken together, this may explain why the 12 month

ACI biopsy assessed in our study contained fewer GAGs than the 20 month and the age-matched controls.

A large body of work has described the relevance of mechanical loading of articular cartilage[39-41]. Mechanical loading maintains the integrity of articular cartilage; however, both disuse and overuse can result in cartilage changes[39]. At the molecular level, chondrocytes are known to respond to mechanical load by altering their gene expression[40]. At the cellular level, mechanical load is known to produce changes in CS chain length, sulphation patterns and their concentrations[41]. For example, in horses, CS chain length can be increased by exercise[42]. At our centre, ACI patients are given a detailed post-operative rehabilitation protocol which includes exercises designed to improve weight bearing, range of motion and strength[43]. In our previously published work[8], the CS chains were considerably shorter in the age-range of ACI biopsies that we examined. In this new study, CS chain length doubled from 12 months (3.91±0.46kDa) to 20 months post ACI (6.92±0.71kDa). Despite this finding, CS chain length was still considerably less than age-matched controls. Previous work has shown that the presence of GalNAc or GalNAc6S can facilitate elongation of the CS chain and the presence of GalNAc4S can prevent elongation of the CS chain[44]. In our study, both the ACI repair tissue and the control tissues had no GalNAc4S but did have low levels of GalNAc and GalNAc6S at the end of their CS chains. Thus one would expect the CS chain length to be longer. It is possible that there was insufficient mechanical load applied to the repair site by the ACI patient as he recovered and that this resulted in the shorter CS chains.

In our previous work, there was less KS in the 12 month ACI repair tissue and it was more sulphated when compared to the control tissue[8]. In our present study, the patients KS levels were initially low and only showed a slight improvement over time. Gal6S-GlcNAc6S was present in the highest concentration in all controls and it doubled in concentration as the ACI tissue matured. In general, all cultured cells tend to produce KS at a reduced rate with less sulphation because KS-specific sulfotransferases are generally down-regulated[45]. All sulphated GAGs are known to interact with collagens to mediate the organisation of the extracellular matrix. In culture, the KS levels may initially be low because there is insufficient collagen. This may also be true for KS levels within the ACI repair tissue.

Following ACI, maturation of the cartilage is known to occur through several steps. Our results agree with other published studies which have shown that ACI tissue needs time to remodel and continues to improve in quality with time[3]. However, even after 20 months the ACI repair tissue has fewer GAGs with different sulphation patterns than controls. Little is known about the mechanisms regulating the biosynthesis of GAG fine structure. Thus there is still a short fall in our biochemical understanding of both GAGs and their role in maturing ACI repair tissue.

Acknowledgement

We would like to thank Mrs Helena Evans and Mrs Janis Menage (RJAH) for help with the biopsies.

Funding

This work was funded by Arthritis Research UK (16181).

References

1. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med.* 1994;331:889-895.

2. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin Orthop Relat Res.* 2000;(374):212-234.

3. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med*. 2010;38(6):1117-24.

4. Ashvin K. Dewan, Matthew A. Gibson, Jennifer H. Elisseeff, Michael E. Trice. Evolution of Autologous Chondrocyte Repair and Comparison to Other Cartilage Repair Techniques. Biomed Res Int. 2014; 2014: 272481

5. Gerjo J V M Van Osch, Mats Brittberg, James E Dennis, Yvonne M Bastiaansen-Jenniskens, Reinhold G Erben, Yrjö T Konttinen, Frank P Luyten. Cartilage repair: past and future – lessons for regenerative medicine. J Cell Mol Med. 2009 May; 13(5): 792–810.

6. Clinical Outcome of Autologous Chondrocyte Implantation Is Correlated With Infrared Spectroscopic Imaging-Derived Parameters. A. Hanifi, J. B. Richardson, J. H. Kuiper, S. Roberts, N. Pleshko Osteoarthritis Cartilage. 2012 Sep; 20(9): 988–996.

7. Vasiliadis HS, Danielson B, Ljungberg M, McKeon B, Lindahl A, Peterson L. Autologous chondrocyte implantation in cartilage lesions of

the knee: long-term evaluation with magnetic resonance imaging and delayed gadolinium-enhanced magnetic resonance imaging technique. *Am J Sports Med*. 2010;38(5):943-949.

8. Sharma A, Wood LD, Richardson JB, Roberts S, Kuiper NJ. Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage. *Arthritis Res Ther.* 2007;9(4):R79.

9. Minas T. Chondrocyte implantation in the repair of chondral lesions of the knee: economics and quality of life. *Am J Orthop* 1998; 27(11):739-744.

10. Van Assche D, Staes F, Van Caspel D, Vanlauwe J, Bellemans J, Saris DB, Luyten FP. Autologous chondrocyte implantation versus microfracture for knee cartilage injury: a prospective randomized trial, with 2-year follow-up. *Knee Surg Sports Traumatol Arthrosc*. 2010;18(4):486-495.

11. Jones DG, Peterson L. Autologous chondrocyte implantation. *J Bone Joint Surg Am.* 2006;88(11):2502-2520.

12. Brun P, Dickinson SC, Zavan B, Cortivo R, Hollander AP, Abatangelo G. Characteristics of repair tissue in second-look and third-look biopsies from patients treated with engineered cartilage: relationship to symptomatology and time after implantation. *Arthritis Res Ther*. 2008;10(6):R132.

13. Krishnan SP, Skinner JA, Bartlett W, Carrington RW, Flanagan AM, Briggs TW, Bentley G. Who is the ideal candidate for autologous chondrocyte implantation? J Bone Joint Surg Br. 2006 Jan;88(1):61-64.

14. José Becerra, José A. Andrades, Enrique Guerado, Plácido Zamora-Navas, José M. López-Puertas and A. Hari Reddi. Articular cartilage: structure and regeneration. *Tissue Engineering Part B: Reviews.* 2010; 16(6): 617-627.

15. Kramer K. Specific sides to multifaceted glycosaminoglycans are observed in embryonic development. *Seminars in Cell & Developmental Biology* 2010;21:631–637.

16. Huckerby TN, Lauder RM, Brown GM, Nieduszynski IA, Anderson K, Boocock J, Sandall PL, Weeks SD. Characterization of oligosaccharides from the chondroitin sulfates. (1)H-NMR and (13)C-NMR studies of reduced disaccharides and tetrasaccharides. *Eur J Biochem*. 2001;268(5):1181-1189.

17. Yamada S, Onishi M, Fujinawa R, Tadokoro Y, Okabayashi K, Asashima M, Sugahara K. Structural and functional changes of sulfated glycosaminoglycans in Xenopus laevis during embryogenesis. *Glycobiology*. 2009;19(5):488-498.

18. West LA, Roughley P, Nelson FR, Plaas AK. Sulphation heterogeneity in the trisaccharide (GalNAcSb1,4GlcAb1,3GalNAcS) isolated from the non-reducing terminal of human aggrecan chondroitin sulphate. *Biochem. J.* 1999;342:223-229.

19. Plaas AH, West LA, Midura RJ. Keratan sulfate disaccharide composition determined by FACE analysis of keratanase II and endo-beta-galactosidase digestion products. *Glycobiology* 2001;11(10):779-790.

20. Lauder RM, Huckerby TN, Nieduszynski IA, Plaas AH. Age-related changes in the structure of the keratan sulphate chains attached to fibromodulin isolated from articular cartilage. *Biochem J* 1998;330:753-757.

21. Bayliss MT, Howat S, Davidson C, Dudhia J. The organization of aggrecan in human articular cartilage. Evidence for age-related changes in the rate of aggregation of newly synthesized molecules. *J Biol Chem* 2000;275(9):6321-6327.

22. Kuiper NJ, Sharma A. A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies. *Osteoarthritis Cartilage*. 2015 Dec;23(12):2233-2241.

23. Holmes MW, Bayliss MT, Muir H. Hyaluronic acid in human articular cartilage. Age-related changes in content and size. *Biochem J* 1988; 250(2):435-441.

24. Roberts S, McCall IW, Darby AJ, Menage J, Evans H, Harrison PE, Richardson JB. Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology. *Arthritis Res Ther*. 2003;5(1):R60-73.

25. Calabro A, Hascall VC, Midura RJ. Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage. *Glycobiology* 2000;10(3):283-293.

26. Plaas AH, West L, Midura RJ, Hascall VC. Disaccharide composition of hyaluronan and chondroitin/dermatan sulfate. Analysis with fluorophore-assisted carbohydrate electrophoresis. *Methods Mol Biol* 2001;171:117-128.

27. Karousou E, Asimakopoulou A, Monti L, Zafeiropoulou V, Afratis N, Gartaganis P, Rossi A, Passi A, Karamanos NK. FACE analysis as a fast and reliable methodology to monitor the sulfation and total amount of chondroitin sulfate in biological samples of clinical importance. Molecules. 2014 Jun 12;19(6):7959-7980.

28. McCarthy HS, Roberts S. A histological comparison of the repair tissue formed when using either Chondrogide(®) or periosteum during autologous chondrocyte implantation. *Osteoarthritis Cartilage*. 2013 Dec;21(12):2048-2057.

29. Roberts S, Menage J, Sandell LJ, Evans EH, Richardson JB. Immunohistochemical study of collagen types I and II and procollagen IIA in human cartilage repair tissue following autologous chondrocyte implantation. Knee. 2009 Oct;16(5):398-404.

30. Niethammer TR, Limbrunner K, Betz OB, Gülecyüz MF, Pietschmann MF, Feist M, Müller PE. Int Orthop. Analysis of the autologous chondrocyte quality of matrix-based autologous chondrocyte implantation in the knee joint. *International Orthopaedics (SICOT)*. 2016 Jan;40(1):205-212.

31. Prydz K. Determinants of glycosaminoglycan structure. *Biomolecules.* 2015 Aug 21;5(3):2003-2022.

32. Kameda T, Koike C, Saitoh K, Kuroiwa A, Iba H. Analysis of cartilage maturation using micromass cultures of primary chondrocytes. *Dev Growth Differ.* 2000 Jun;42(3):229-236.

33. Williams GM, Klisch SM, Sah R. Bioengineering Cartilage Growth, Maturation, and Form. *Pediatric Research* 2008;63:527–534.

34. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P. Re-expression of cartilage-specific genes by re-differentiated human articular chondrocytes cultured in alginate beads. *Exp Cell Res.* 1994;212(1):97-104.

35. Kolettas E, Buluwela L, Bayliss MT, Muir H. Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. *Journal of Cell Science* 1995;108:1991-1999.

36. Harrison PE, Ashton IK, Johnson WE, Turner SL, Richardson JB, Ashton BA. The in vitro growth of human chondrocytes. *Cell Tissue Bank*. 2000;1(4):255-260.

37. Liu H, Lee YW, Dean MF. Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. *Biochim Biophys Acta*. 1998;1425(3):505-15.

38. Maroudas A, Bayliss MT, Uchitel-Kaushansky N, Schneiderman R, Gilav E. Aggrecan Turnover in Human Articular Cartilage: Use of Aspartic

Acid Racemization as a Marker of Molecular Age. *Arch Biochem Biophys.* 1998;350:61-71.

39. Kobayashi S, Meir A, Urban J. Effect of cell density on the rate of glycosaminoglycan accumulation by disc and cartilage cells in vitro. *J Orthop Res*. 2008 Apr;26(4):493-503.

40. Sun HB. Mechanical loading, cartilage degradation, and arthritis. *Ann N Y Acad Sci*. 2010;1211:37-50.

41. Wang QG, Nguyen B, Thomas CR, Zhang Z, El Haj AJ, Kuiper NJ. Molecular profiling of single cells in response to mechanical force: comparison of chondrocytes, chondrons and encapsulated chondrocytes. *Biomaterials*. 2010;31(7):1619-1625.

42. Brown MP, Trumble TN, Plaas AH, Sandy JD, Romano M, Hernandez J, Merritt KA. Exercise and injury increase chondroitin sulfate chain length and decrease hyaluronan chain length in synovial fluid. *Osteoarthritis Cartilage*. 2007 Nov;15(11):1318-25.

43. Bailey A, Kuiper (nee Goodstone) NJ, Roberts S, Hughes J, Roberts S, Niekerk L, Richardson JB, Rees D. Rehabilitation after Oswestry autologous-chondrocyte implantation: the OsCell protocol. *J Sport Rehabil*. 2003;12:104-118.

44. Roden L. Structure and metabolism of connective tissue proteoglycans. In: Lennarz W, Ed. The Biochemistry of Glycoproteins and Proteoglycans. New York: Plenum Press 1980:286-306.

45. K Kitayama, Y Hayashida, K Nishida, TO Akama, Enzymes responsible for synthesis of corneal keratan sulfate glycosaminoglycans. *J Biol Chem*. 2007;282:30085–30096.

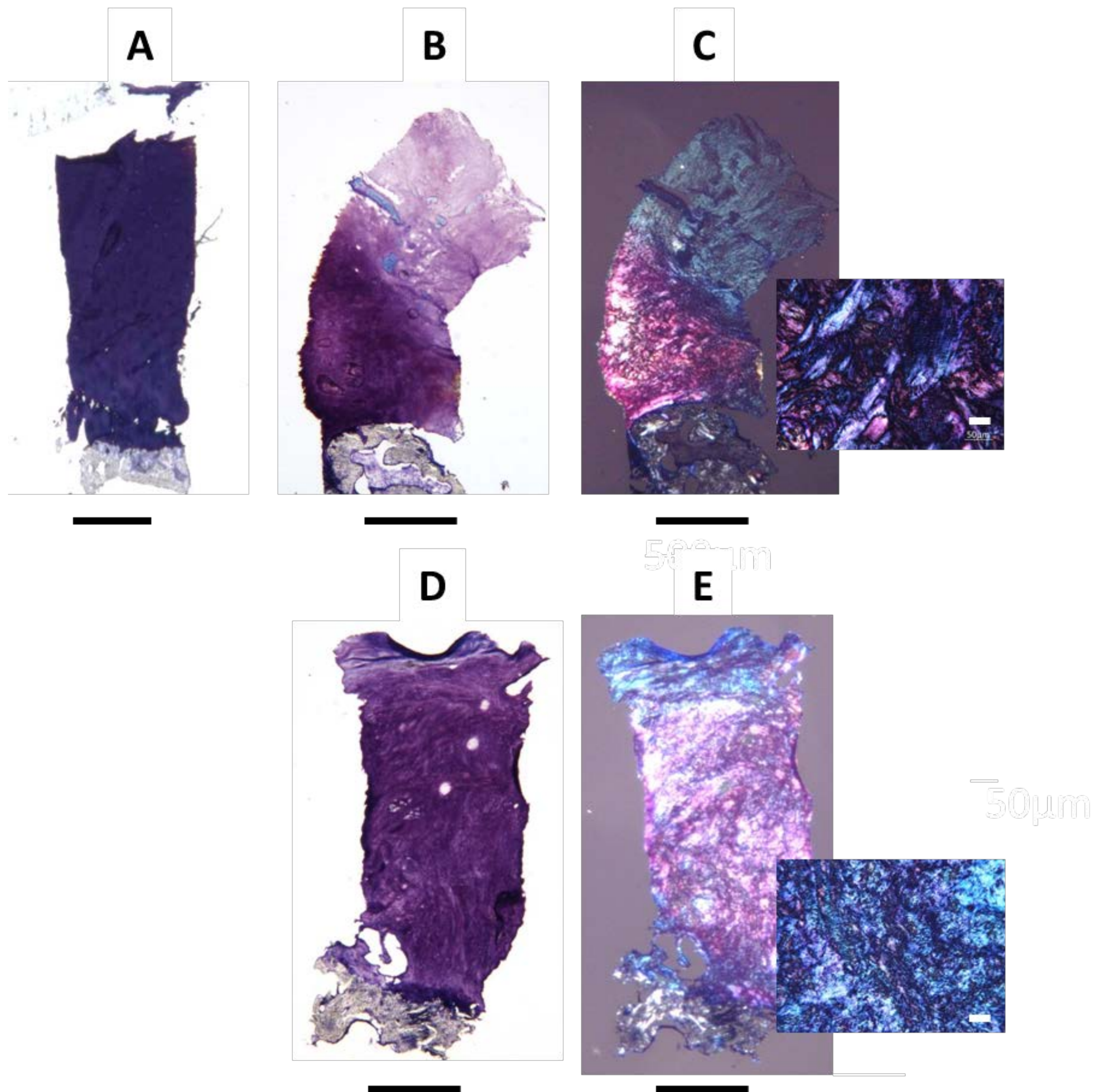

Figure 1: Representative histological images of full depth biopsies from the medial femoral condyles of a 22 year old cadaveric sample and a 25 year old post implantation autologous chondrocyte implantation (ACI) patient. Tissue is stained with toluidine blue. Histological analysis of (A) the 22 year old cadaveric control, (B) the 25 year old patient at 12 months post ACI, (C) polarised light image of the tissue at 12 month including a high power image, (D) the 25 year old patient at 20 months post ACI, and (E) polarised light image of the tissue at 20 months including a high power image. Scale bars are 500µm for the full length biopsies and 50µm for the insert images.

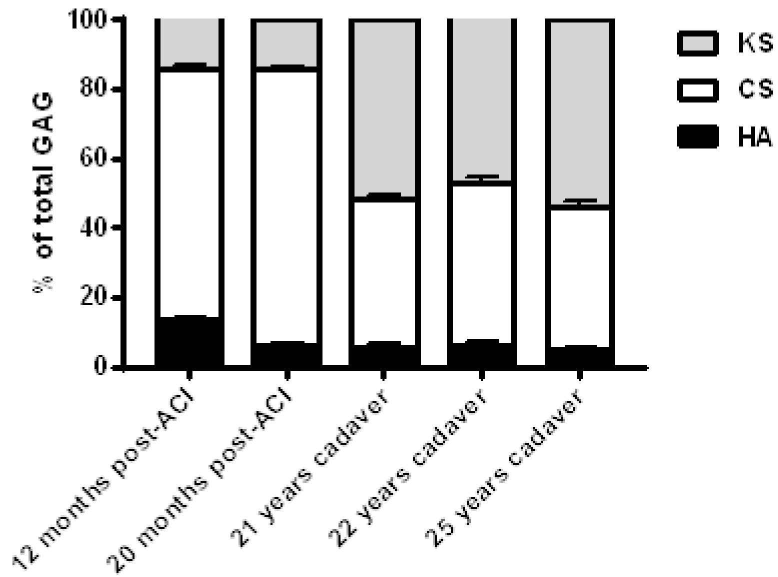

Figure 2: The percentage of HA, CS, and KS in cartilage biopsies taken from an ACI patient and three age matched cadaveric knees (aged 21-25 years). Data are expressed as a percentage of the total GAG. The data are represented as the mean ± SEM (n=8).

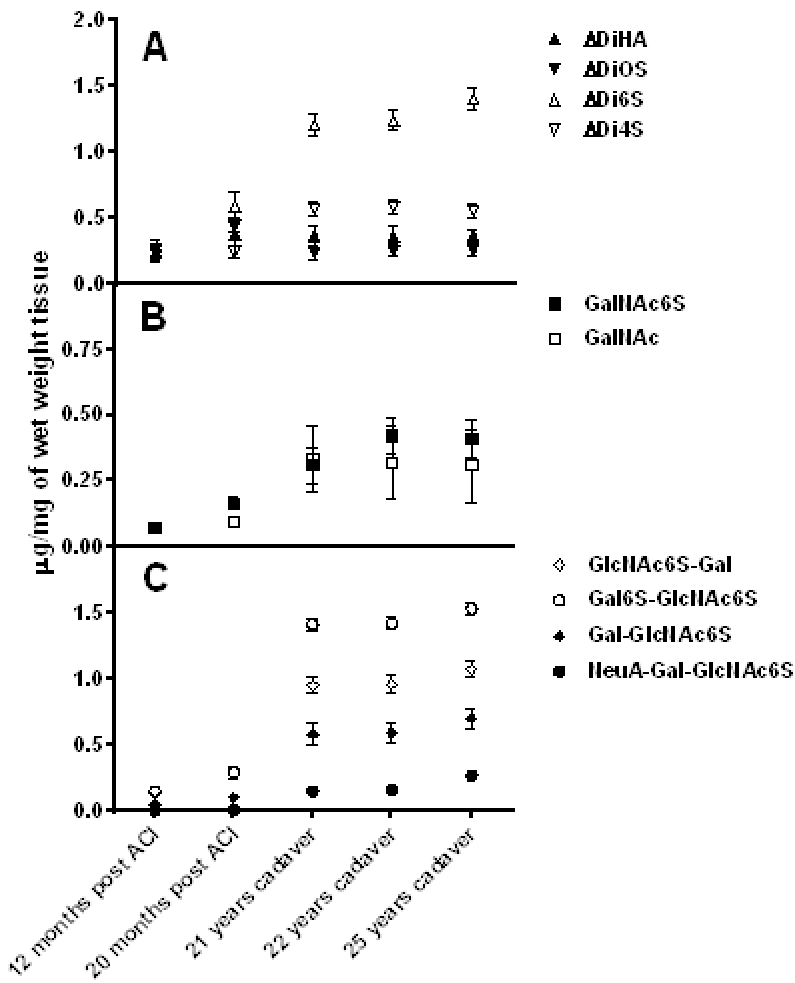

Figure 3: Quantitation of levels of (A) HA and CS disaccharides, (B) CS nonreducing termini, and (C) KS disaccharides in both ACI repair and cadaveric tissues. Data are expressed as micrograms of disaccharide per milligram of dry weight of cartilage tissue. The data are represented as the mean ± SEM (n=8).

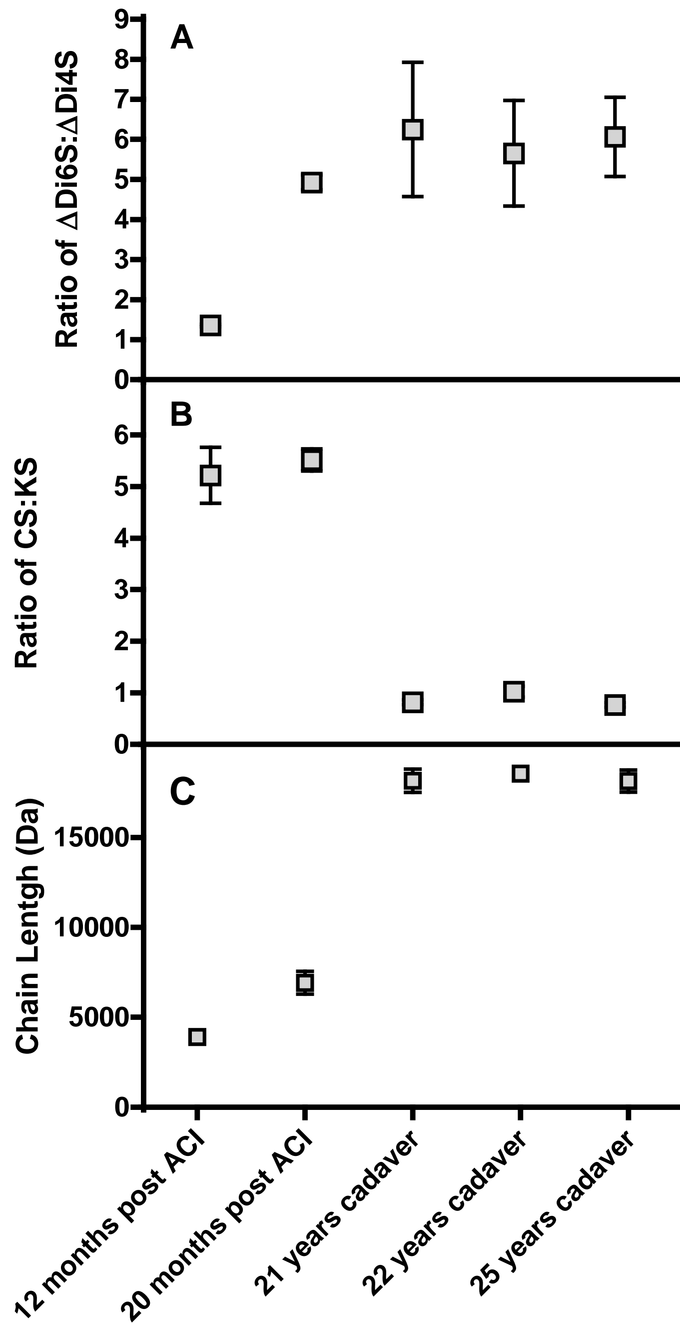

Figure 4: Comparison of the (A) ΔDi6S:ΔDi4S ratios, (B) CS:KS ratios, and (C) CS chain length in ACI repair and cadaveric tissues. The data are represented as the mean ± SEM (n=8).