Osteoarthritis and Cartilage xxx (2015) 1–9

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies

N.J. Kuiper † *, A. Sharma ‡

† *Institute for Science & Technology in Medicine (ISTM), University of Keele, Staffordshire, ST5 5BG, UK*
‡ *Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA*

ARTICLE INFO

Article history:
Received 15 May 2015
Accepted 7 July 2015

Keywords:
Human
Fluorophore-assisted carbohydrate electrophoresis
Glycosaminoglyans
Knee
Articular cartilage
Cartilage regeneration

SUMMARY

Objective: Ideally, cartilage regenerative cell therapy should produce a tissue which closely matches the microstructure of native cartilage. Benchmark reference information is necessary to assess the quality of engineered cartilage. Our goal was to examine the variation in glycosaminoglycans (GAGs) in cartilage zones within human knee joints of different ages.

Design: Osteochondral biopsies were removed from the medial femoral condyles of deceased persons aged 20–50 years. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) was used to profile GAGs through the superficial, middle and deep zones of the articular cartilage. Differences were identified by statistical analysis.

Results: Cartilage from the younger biopsies had 4-fold more hyaluronan in the middle zone than cartilage from the older biopsies. The proportion of hyaluronan decreased with increasing age. Cartilage from the middle and deep zones of younger biopsies had significantly more chondroitin sulphate and keratan sulphate than the cartilage from older biopsies. This would suggest that chondrocytes synthesise more sulphated GAGs when deeper in the tissue and therefore in conditions of hypoxia. With increasing age, there was significantly more chondroitin-6 sulphate than chondroitin-4 sulphate. For the first time, unsulphated chondroitin was detected in the superficial zone.

Conclusions: As an outcome measure, FACE offers the potential of a complete, detailed assessment of all GAGs and offers more information that the widely used 1,9-dimethylmethylene blue (DMMB) dye assay. FACE could be very useful in the evolving cartilage regeneration field.

Introduction

Adult articular cartilage is a complex anisotropic tissue with a limited capacity for self-repair. Cartilage regenerative cell therapy was first introduced to treat isolated defects of articular cartilage[1] which if left untreated are likely to progress to osteoarthritis[2]. The cell therapy field has continued to evolve and now there is hope of providing long term benefit to individuals with more extensive damage and even osteoarthritis[3]. The ultimate goal of cell therapy is to create tissue which closely mimics the microstructure found in native articular cartilage. If we are to make meaningful assessments of cartilage generated through cell therapy then the field needs detailed benchmarks concerning the extracellular matrix (ECM) components of native articular cartilage.

The ECM is organised into distinct zones; superficial, middle and deep[4,5]. As a result of differences in chondrocyte activity and mechanical loading, the ECM composition and its structure varies greatly between each zone[6]. In brief, collagen fibres in the superficial zone are densely packed and oriented parallel to the articular surface, in the middle zone they have a random orientation and in the deep zone they are oriented perpendicularly to the surface. Proteoglycan concentration is greatest in the middle zone. Proteoglycans and glycosaminoglycans (GAGs) are critical to the function of articular cartilage[7,8]. With the exception of hyaluronan (HA), GAGs are synthesised covalently bound to core proteins to

* Address correspondence and reprint requests to: N.J. Kuiper, Institute for Science and Technology in Medicine (ISTM), University of Keele, The Robert Jones and Agnes Hunt Orthopaedic Hospital, The Arthritis Research Centre, Oswestry, SY10 7AG, UK.

E-mail addresses: n.j.kuiper@keele.ac.uk (N.J. Kuiper), as3182@cumc.columbia.edu (A. Sharma).

http://dx.doi.org/10.1016/j.joca.2015.07.011

form proteoglycans such as aggrecan[8,9]. The highly anionic GAGs endow cartilage with its resistance to compressive loading by binding and organising water molecules[4]. Both the proteoglycan core proteins and their associated GAGs have important functional roles in tissue remodelling[10], up-take of proteins[11], intracellular signalling[12] and cell migration[13]. These functions depend largely on their post-translational modifications and the protein cores to which they are attached. To date, the size and distribution of the proteoglycans have been well studied in whole tissue[7]. However there is a paucity of information detailing the GAGs and even less information about their variation through full-depth human articular cartilage. Stockwell and Scott[14] were the first to use a combination of histochemical and biochemical methods to show that GAG concentration varied through the full-depth human articular cartilage. Since then several investigators have confirmed their results using immunological and biochemical approaches in a range of different species[15–18]. There are excellent immunological approaches to study GAG distribution in tissue samples[15] but these approaches have a more limited use in the characterisation and quantitation of GAGs. Most biochemical GAG assays require large amounts of tissue or cells and do not differentiate between GAG types[16,17]. For example, the widely used 1,9-dimethylmethylene blue (DMMB) dye assay[18], is sensitive to 1 μg of total sulphated GAG but it cannot differentiate GAGs nor detect unsulphated HA. There are more sensitive and discriminatory approaches such as fluorophore-assisted carbohydrate electrophoresis (FACE)[19–22]. FACE can discriminate between GAGs in small extracts of tissue or cells[19,20], for example in our laboratory, we have used FACE to analyse GAG profiles in as little as 5 μg of cartilage[21] or around 25,000–50,000 freshly isolated chondrocytes or chondrons[22]. As an outcome measure, FACE offers the potential of a complete, detailed assessment of GAGs which could be very useful in the evolving cartilage regenerative cell therapy field.

There are three classes of GAGs in cartilage; hyaluronan (HA), chondroitin sulphate (CS) and keratan sulphate (KS). HA accounts for 1–10% of GAGs in articular cartilage and is composed of GlcA and GlcNAc linked by a β1,3-glycosidic bond. Previously, using full-depth cartilage, we have shown that HA content decreases with increasing age[21]. Other published studies using full-depth human cartilage demonstrated that with increasing age, the molecular mass of HA decreased but there was a slight increase in HA concentration[23,24]. To date, there are no reports on the absolute quantities of HA within the superficial, middle and deep zones of aging cartilage. Almost 80% of GAGs in adult articular cartilage are CS chains. They are composed of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). Each monosaccharide may be left unsulphated, sulphated once, or sulphated twice. As a function of age, there is increased sulphation at the C6 sites relative to the C4 sites[16,21]. The monosaccharides are linked by a β1,3 glycosidic bond and the chain can be terminated with either GalNAc4S (in which S stands for sulphate), GalNAc6S or GalNAc4,6S[21,25]. It is still not clear why there is such variation in the terminal sugar. KS chains represent 5–20% of the GAGs in articular cartilage. KS is composed of galactose (Gal) and N-acetylglucosamine (GlcNAc) linked by a β1,4 glycosidic bond[26,27]. KS can be branched. KS chains can be modified by O-sulphation of the hydroxyl groups at C6 of both the Gal and/or the GlcNAc, resulting in unsulphated, monosulphated or disulphated repeat disaccharides. KS chains demonstrate an age-related increase in chain length and sulphation, and the amount of KS has been shown to increase progressively through the full-depth of cartilage[7].

A small number of studies have assessed differences in chondrocyte phenotype[5], matrix morphology[6] and compressive properties[28] through the full-depth of articular cartilage. To date there are only a small number of reports describing GAG variation through the full-depth of articular cartilage or with increasing age[7,16,17,23,29]. Most of these studies do not report absolute HA, CS or KS concentrations. Most cartilage regeneration strategies are focussed on “one size fits all” which does not take into account the potential variations within the cartilage microanatomy. A tissue engineered construct might be suitable for implantation into a 30 year olds knee but not necessarily a 50 year old knee. With a view towards this we have utilised the well-known technique of FACE[19–22] to profile GAGs in articular cartilage obtained from a well-defined site in the adult knee joint. The overall goal was to quantify and compare both the distribution and the absolute concentrations of HA, CS and HA in articular cartilage obtained from the medial femoral condyles in an age-range of macroscopically normal adult human knees. Comparisons were made through the full-depth of the tissue. Our intention is that the information obtained will provide a valuable benchmark for cartilage regenerative strategies. Further, that FACE becomes an additional outcome measure to support existing immunological and DMMB assay data.

Materials and methods

Biopsies of cadaveric human knees

Four cadaveric knees (22, 30, 40 & 50 years old) were obtained within 24 h of death from the UK Human Tissue Bank with approval by the Trent Research Ethics Committee, UK. Subjects were victims of road accidents and had not suffered with acute or chronic disease. All knee joints appeared healthy and intact apart from the 50-year old whose articular cartilage showed slight surface fibrillation. From each joint, nine osteochondral biopsies (1.8 mm in diameter, ~5 mm in height) were taken perpendicularly from the articulating surface through the full depth of cartilage and subchondral bone of the medial femoral condyles.

Treatment of biopsies

One unfixed biopsy was directly sectioned at 5 μm with a conventional ultramicrotome and a glass knife. Under a dissection microscope, the thickness of hyaline articular cartilage from the articular surface to the cartilage–bone interface was measured and used to define three zones: superficial (~10% of thickness), middle (~10%), and deep (~80%). The subchondral bone from the remaining eight biopsies was carefully removed after scoring along the cartilage–bone interface. The articular cartilage was further divided into the superficial, middle, and deep zones with a scalpel under a dissection microscope. Each piece of tissue was snap frozen in liquid nitrogen-cooled hexane and stored in liquid nitrogen until studied.

Preparation of GAG saccharides for FACE

GAGs were ethanol extracted from each portion of biopsy using our previously published methodology[21,22]. Purified GAG chains were cleaved using GAG-specific enzymes (Seikagaku). Briefly, HA was digested into disaccharides (ΔDiHA) using 100 mU/ml of hyaluronidase *Streptococcus dysgalactiae* for 1 h at 37°C[21]. CS was digested into disaccharides (6-sulphated, ΔDi6S; 4-sulphated, ΔDi4S; and unsulphated, ΔDi0S) with 100 mU/ml of chondroitinase ABC (cABC) for 3 h at 37°C. Sulphation 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. The non-reducing terminal sugars of CS were identified by mercuric ion treatment. KS was digested as previously described[19–22]. KS digestion was assessed by sequential digestion with 100 mU/ml of keratanase II for 3 h and 100 mU/ml of endo-β-galactosidase for

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

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. This approach will generate five digestion products from the internal KS chain (unsulphated disaccharide, GlcNAcβ1,3Gal; monosulphated disaccharide GlcNAc6Sβ1,3Gal; fucosylated trisaccharide, Galβ1,2 [fucα1,3]GlcNAc6S; monosulphated disaccharide, Galβ1,4GlcNAc6S; disulphated disaccharide, Gal6Sβ1,4glcNAc6S) and one digestion product which caps the KS chain (disulphated trisaccharide NeuAα2,3Gal6Sβ1,4GlcNAc6S).

Fluorotagging of cleaved GAGs for separation on FACE gels

Lyophilised enzyme-digested cleaved GAG samples and predefined saccharide standards (Seikagaku) were reconstituted with the fluorescent tag, 5 μl 12.5 mM 2-aminoacridone, in glacial acetic acid/DMSO (3:17, v/v) and incubated at room temperature for 15 min. Five microlitres of 1.25 M sodium cyanoborohydride in distilled deionised water were added. Samples were incubated at 37°C for 16 h. After tagging, 10 μl of 25% glycerol were used to quench excess sodium cyanoborohydride. Electrophoresis on monosaccharide FACE gels was carried out for 80 min at 4°C.

FACE gel imaging & quantitation of GAGs

Gels were placed on a transilluminator light box fitted with a 312 nm light source. Fluorescent images were captured using a GelDoc-It High CCD Camera (12-bit depth, UVP, Cambridge, UK) and the mean pixel density for each product band was quantified using LabWorks Software (UVP). For each gel, FACE product bands were identified by their co-electrophoresis with a range of pre-defined saccharide fluorotagged standards. Two image exposures were captured. The first exposure was used for quantitation as it had all of the pixels within a linear 12-bit depth range to provide base line data and the second exposure over-saturated pixel intensity to allow identification of less abundant structures. Accurate quantitation was achieved between 10 and 400 pmole of product. Quality control was rigorously and routinely performed using a mixture of a known concentration of fluorotagged saccharide standards and enzyme-digested appropriate substrate controls. To control for gel-to-gel variation one cartilage sample was repeated when running multiple gels.

Statistical analysis

Results for the eight biopsies are expressed as the mean concentration (g/L) and their 95% confidence intervals (CI). Two-way analysis of variance (ANOVA), followed by a Tukey-Honest Significant Difference (HSD) post hoc test was performed to determine zone and age dependent differences in GAG content. For all analyses a p-value below 0.05 was assumed to denote statistical significance. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA).

Results

FACE analysis of hyaluronidase and chondroitinase digestion products

Figure 1 is a representative FACE gel showing the co-electrophoresis of a known concentration of monosaccharide and disaccharide fluorotagged standards alongside hyaluronidase and chondroitinase digestion products isolated from a 30 year old ($n = 8$) and a 40 year old ($n = 8$). GAG-specific enzymes were used at a concentration and a time which is known to completely depolymerise the HA and CS[21,22]. Digital images for each gel were taken at two exposures. The image in Fig. 1A has all pixels within a 12-bit depth range and was used for quantification. The image in Fig. 1B has oversaturated pixel intensity and was used to identify any less abundant structures. All cartilage samples contained HA disaccharides (ΔDiHA) and CS disaccharides (ΔDi0S, ΔDi4S and ΔDi6S). No other disaccharide bands were observed. Data to confirm the terminal sugars and sulphation of the CS and are not shown.

Hyaluronan (HA) disaccharide analysis

The quantities of total HA disaccharides (ΔDiHA) in each zone are shown in Fig. 2. HA was detected in all cartilage zones and in all

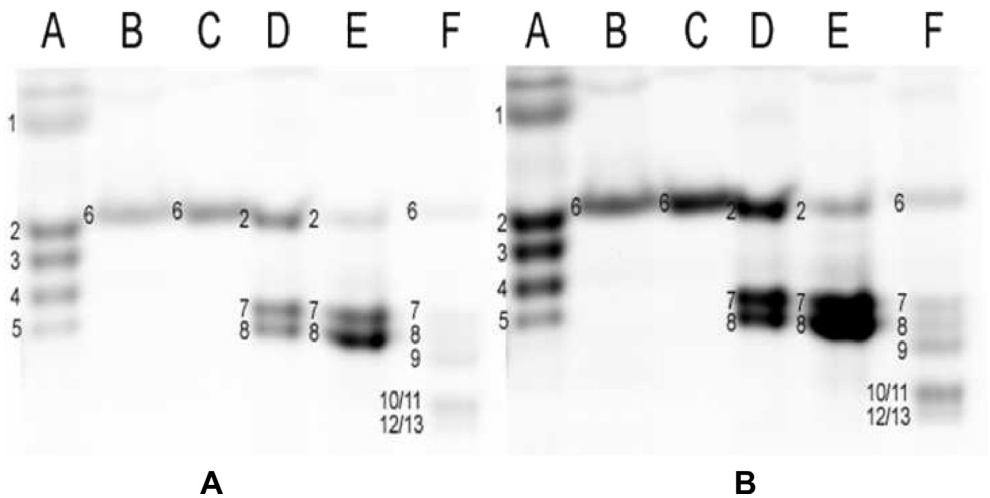

Fig. 1. A representative FACE gel of hyaluronidase and chondroitinase digestion products obtained from the superficial zones of a 30 year old biopsy (***Lanes B & E***) and a 40 year old biopsy (***Lanes C & D***). The first exposure (**1A**) was used for quantitation to provide baseline data. The second exposure (**1B**) had over-saturated pixel intensity to allow identification of less abundant structures. Lanes A and F contain a mixture of pre-defined fluorotagged saccharide standards. ***Lane A***: (1) N-acetyl galactosamine (galNAc), (2) unsulphated chondroitin disaccharides (ΔDiOS), (3) N-acetyl galactosamine-6 sulphate (6S-galNAc), (4) N-acetyl galactosamine-4 sulphate (4S-galNAc), and 4-/6-sulphated N-acetyl galactosamine (4,5S-galNAc). ***Lane F***: (6) hyaluronan disaccharides (ΔDiHA), (7) chondroitin 6-sulphate disaccharides (ΔDi6S), (8) chondroitin 4-sulphate disaccharides (ΔDi4S), (9) dermatan sulphate disaccharides (ΔDi2S), (10/11) ΔDi4,6S, (12/13) ΔDi2,6S. Cadaveric biopsies were digested with hyaluronidase *Streptococcus dysgalactiae* SD (***Lane B & C***), chondroitinase ABC (***Lane D & E***) chondroitinase ABC. The migratory positions of (6) ΔDiHA, (2) ΔDiOS, (7) ΔDi6S and (8) ΔDi4S are also shown.

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

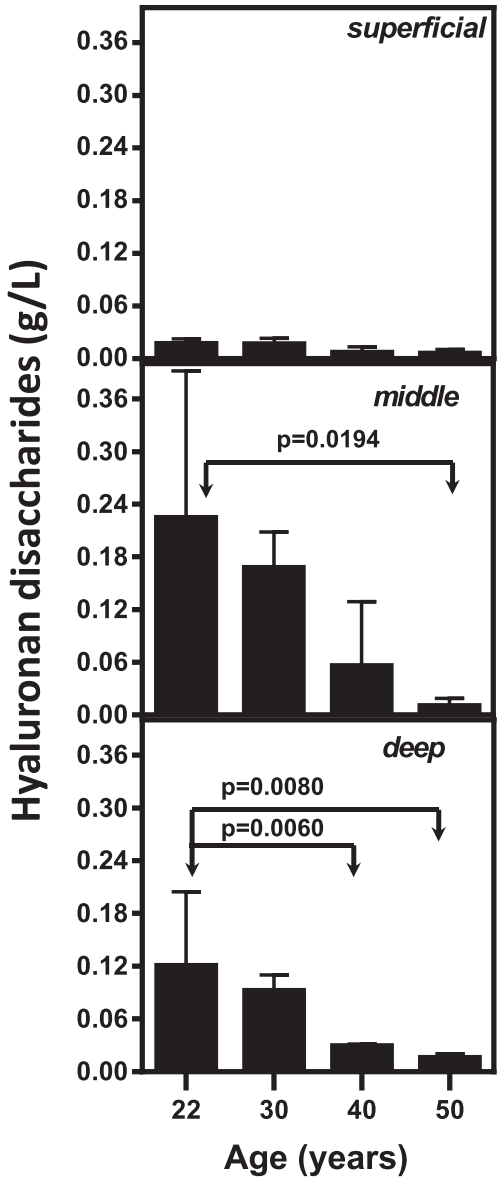

Fig. 2. Hyaluronan (HA) disaccharide concentration (g/L) within the superficial, middle and deep zones of an age range (22, 30, 40 & 50 years) of human articular cartilage biopsies. The graph illustrates the mean concentration (g/L) ± 95% confidence limits (CL) for the eight biopsies. Zones with significantly different HA content when compared between ages are indicated by arrows on the associated horizontal bars.

ages. The cartilage biopsies ($n = 8$) from the 22 year old had the highest amount of HA (full-depth mean 0.371, 95% CI 0.273,0.483 g/L) whilst the cartilage biopsies ($n = 8$) from the 50 year old had the lowest amount of HA (full-depth mean 0.031, 95% CI 0.026,0.039 g/L). Overall, the mean concentration of HA was highest in the middle zone of the cartilage biopsies. The mean concentration of HA was significantly higher in the middle and deep zones of the cartilage biopsies obtained from the 22 year old compared with the 40 year old. Within all cartilage zones the concentration of HA content decreased with age.

Chondroitin sulphate (CS) disaccharide analysis

The quantities of total CS disaccharides (ΔDi0S, ΔDi4S and ΔDi6S) in each zone are shown in Fig. 3A. For the 22 and 30 year olds, the mean concentration of CS was significantly higher in the middle and deep zones of the cartilage compared with the superficial zone (22 year old; middle zone $p = 0.027$, deep zone $p = 0.001$ & 30 year old; middle zone $p = 0.001$, deep zone $p = 0.008$). Overall total CS concentration (g/L) decreased with increasing age. Table I illustrates the distribution of ΔDi0S%, ΔDi4S% and ΔDi6S% across the zones and ages. ΔDi0S decreased with age, with little or no ΔDi0S detected in the 40 and 50 year old subjects. As shown in Fig. 3B, ΔDi6S% increased and ΔDi4S% decreased with increasing age. There was significantly more ΔDi6S than ΔDi4S in the middle zone and deep zones for all tissue ages.

Identification of non-reducing terminal structures on CS

We were able to identify two non-reducing terminal sugars; GalNAc4S and GalNAc6S. Table II shows the total mean concentration (g/L) of GalNAc4S and GalNAc6S for each biopsy. The chain terminal residues of adult cartilage CS clearly change with age. CS chains from the 22 and 30 year old biopsies had more GalNAc6S than GalNAc4S non-reducing termini. CS chains from the 40 and 50 year old biopsies had less GalNAc6S in comparison to the 22 and 30 year old biopsies. Statistical analysis using Tukey-Honest HSD confirmed that there was significantly more GalNA6S than GalNAc4S in the deep zone in all ages (22 years $p = 0.002$, 30 years $p = 0.001$, 40 years $p = 0.002$ and 50 years $p = 0.008$).

Proportion of KS in the biopsies

The quantities of total KS saccharides in each zone are shown in Fig. 4. The superficial zone contained very little KS whilst, for all ages, KS content was highest in the deep zone. The mean concentrations (g/L) of all KS digestion products detected by FACE are shown in Table III. KS digestion generated one digestion product which caps the KS chain (disulphated trisaccharide NeuAcα2,3Galβ1,4GlcNAc6S) and five digestion products from the internal KS chain (unsulphated disaccharide, GlcNAcβ1,3Gal; monosulphated disaccharide GlcNAc6Sβ1,3Gal; fucosylated trisaccharide, Galβ1,2[fucα1,3]GlcNAc6S; monosulphated disaccharide, Galβ1,4GlcNAc6S; disulphated disaccharide, Gal6Sβ1,4glcNAc6S). The monosulphated disaccharide, Galβ1,4GlcNAc6S and the disulphated disaccharide, Gal6Sβ1,4glcNAc6S were present through the full-depth at all ages. Gal6Sβ1,4GlcNAc6S made up the majority of the internal KS chains in all ages. Galβ1,2[fucα1,3]GlcNAc6S was absent in the 22 and 30 year old biopsies but appeared in the 40 and 50 year old biopsies. Whilst GlcNAcβ1,3Gal was present in the 22 and 30 year old biopsies but absent in the 40 and 50 year old biopsies.

Comparison of the proportions of CS, HA and KS in the biopsies

FACE analysis revealed the differences in the proportions of HA, CS and KS through full-depth cartilage at all ages. These data are summarised in Fig. 5 where each GAG, with its constituent saccharide component, is expressed as a total percentage of GAGs analysed. HA comprised no more that 6% of the total GAGs and was predominantly present in the middle zone of the 22, 30 and 40 year old biopsies. There is a greater proportion of KS to CS in all but the superficial zone of the 40 and 50 year old biopsies.

Discussion

In this study we have focussed on GAGs and their distribution within cartilage since a detailed understanding of native articular cartilage microstructure could provide essential reference points for the evaluation of cartilage cell therapies. This study provides a

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

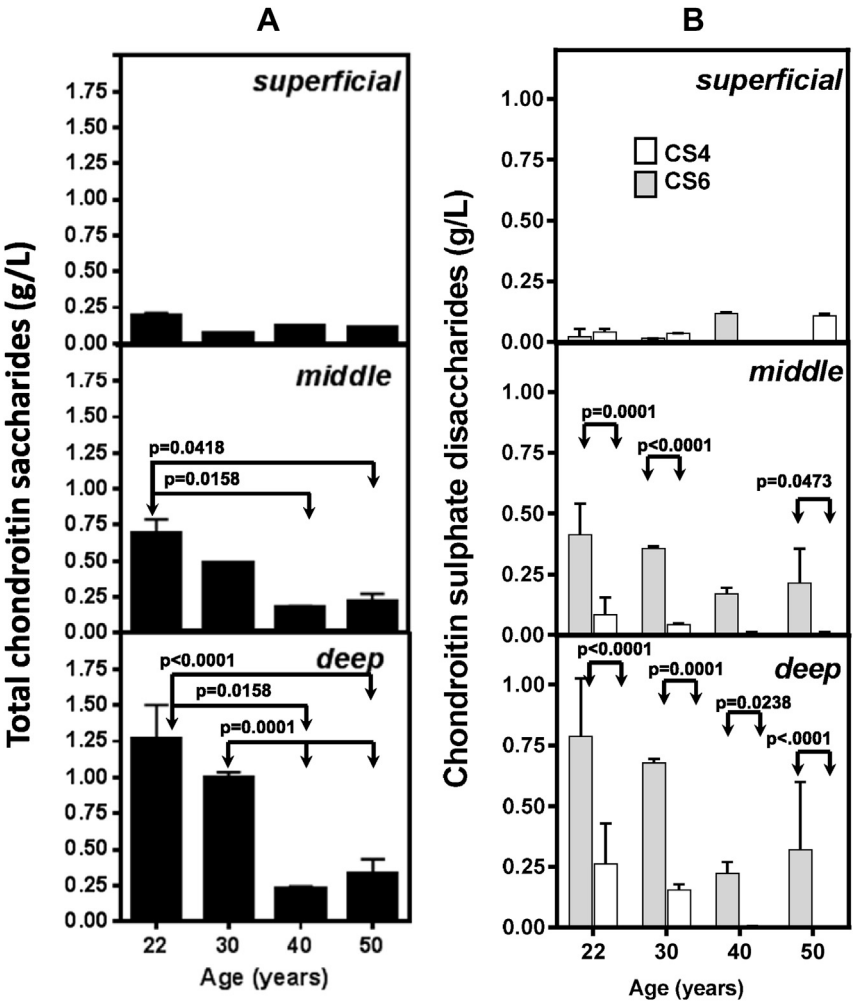

Fig. 3. Total chondroitin disaccharides (CS; ΔDi0S, ΔDi4S and ΔDi6S) concentration (g/L) (**Figure 3A**) and the concentration of chondroitin ΔDi4S and ΔDi6S saccharides (**Figure 3B**) within the superficial, middle and deep zones of an age range (22, 30, 40 & 50 years) of human articular cartilage biopsies. The graph the mean concentration (g/L) ± 95% confidence limits (CL) for the eight biopsies. Zones with significantly different CS disaccharide content when compared with those ages are indicated by arrows on the associated horizontal bars.

powerful, detailed analysis of the variation in GAG saccharides from an age range of cadaveric articular cartilage and to our knowledge there is no other study available providing such insights. One drawback to our study is that FACE only generates mono- and di-saccharides data, and these are difficult to correlate with total cartilage GAG molecules. Another drawback is that we have only sampled one knee joint for each decade (20 years—50 years) and that we should be cautious in how we interpret these data. Despite these two drawbacks, we demonstrate three key points. First, age and zone are important determinants of GAG variation and content. Overall, this is not surprising since many studies have reported differences between zonal chondrocytes in regards to their ECM, cell size, and ECM gene expression[4–7,23,24]. What is of interest is that this detailed understanding of GAGs, on the whole, is not translated into strategies for the regeneration of articular cartilage. Second, the detailed analysis of full-depth cartilage has revealed subtle variations in GAG content and composition which would not be identified by the widely used DMMB assay[18]. It has recently been reported that the DMMB assay can result in an overestimation of sulphated GAGs because of interference from HA and nucleic acids[30]. Thirdly, very little cartilage is required for a complete GAG analysis which is an important consideration.

Table I
The calculated data for all chondroitin saccharide structures (ΔDi0S, ΔDi4S and ΔDi6S), as detected by FACE analysis. Data are represented as a percentage to illustrate the proportion of each saccharide within the superficial, middle and deep zones. Data are shown for each subject aged 22, 30, 40 & 50 years old

Chondroitin saccharide structures	Percentage (%)											
Age (years)	22			30			40			50		
Zone	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep
ΔDi0S	68	28	18	27	18	17	0	1	1	0	0	3
ΔDi4S	10	12	20	21	9	15	98	3	1	2	3	0
ΔDi6S	22	60	62	52	73	68	2	96	98	98	97	97

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

Table II
The calculated data for the non-reducing termini of chondroitin sulphate, as detected by FACE analysis. The values represent the mean concentration (g/L) and their 95% confidence intervals (CI; lower limit, upper limit) of eight biopsies taken from one area. Data are shown for each subject aged 22, 30, 40 and 50 years old. ND represents non detectable by FACE analysis

Chondroitin sulphate disaccharides	Mean concentration (lower limit, upper limit) g/L											
Age (years)	22			30			40			50		
Zone	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep
GalNAc4S	**0.016** (0.015,0.017)	**0.059** (0.055,0.064)	**0.098** (0.092,0.193)	**0.060** (0.056,0.064)	**0.016** (0.015,0.017)	**0.098** (0.091,0.106)	**0.002** (0.001,0.003)	**0.009** (0.008,0.010)	**0.016** (0.014,0.017)	**0.008** (0.007,0.009)	**0.015** (0.009,0.021)	**0.012** (0.010,0.034)
GalNAc6S	**0.103** (0.099,0.107)	**0.108** (0.099,0.117)	**0.206** (0.202,0.209)	**0.020** (0.016,0.024)	**0.202** (0.165,0.239)	**0.383** (0.313,0.545)	ND	**0.007** (0.006,0.008)	**0.125** (0.123,0.126)	ND	**0.004** (0.003,0.005)	**0.107** (0.105,0.108)

The mean concentration is in boldface.

We have shown that HA levels decrease with increasing age and that the middle zone is relatively HA-rich. Our findings agree with previous studies using more laborious and time consuming biochemical approaches and large tissue samples[23,24,29]. For example one previous study[29] reported similar results using a biotinylated hyaluronic acid binding region probe to assess the distribution of HA in full-depth cartilage from subjects aged 19, 30 and 37 years. In cartilage, HA binds and immobilises aggrecan to form massive aggregates with fixed negative charges that retain water and endow the tissue with its critical mechanical properties. Ideally cell based therapies and tissue engineered constructs would need to reach the same or greater concentration of HA within age-matched tissue to match the maturity of native cartilage. Some recent studies have begun to take this into consideration[31–33]. For example, one study[33] used composition-based finite element modelling to demonstrate that by fine tuning the temporal deposition of GAGs they could influence the swelling behaviour and the resultant compressive stiffness of tissue engineered cartilage. Our findings might provide further insights to help achieve an ideal maturation state that is compatible with cartilage integration.

In previous studies, including our own, changes in CS sulphation levels and patterns have been associated with mechanical loading, aging, type of scaffold and chondrocyte metabolic activity[6,16,17,22,33,34]. Using capillary electrophoresis, Bayliss *et al.*[16] showed that from age 20 onwards both ΔDi6S and ΔDi4S levels were relatively constant but their distribution within different knee cartilage zones changed with increasing age. The same study also found that ΔDi0S was constant throughout life. Using the DMMB assay, Rogers *et al.*[17] showed reported higher weight bearing regions of knee cartilage had higher concentrations of sulphated GAGs but they did not assess changes in depth. By contrast to these two studies, we report absolute levels for all CS saccharides within each zone. We found that ΔDi6S% increased and ΔDi4S% decreased with increasing age, and that there were clear differences within the cartilage zones. The functional implication of increased CS-6 in adult native cartilage might be linked to the integrity of the articular cartilage. One could speculate that at implantation it would be better to have the deep and middle zones of the tissue with a higher ratio of CS-6 to CS-4 so that it could immediately match the swelling pressures and cope with variable compressive loads. We found ΔDi0S to be at its highest in the superficial zone in the 22 and 30 year old biopsies and the levels decreased in the older biopsies. The presence of ΔDi0S within the superficial zone is a novel finding. This finding could suggest that there is less 4,6-sulfotransferase activity to produce less or no sulphated CS to reduce swelling pressure within the superficial zone. Alternatively there could be different types of proteoglycans within the superficial zone. This result suggests that it would be ideal to implant a tissue with less sulphated GAG in the superficial zone.

Plaas *et al.*[25] were the first to characterise the non-reducing termini on CS in cartilage tissue. Using high performance liquid chromatography, they determined that the predominant terminal structures were the monosaccharides; GalNAc4S and GalNAc4,6S. They were not able to quantitate GalNAc6S in their study since it co-eluted with another product. Further work by Calabro *et al.*[35] using FACE identified GalNA4S, GalNAc6S and GalNAc4,6S as non-reducing terminal structures on CS. The internal sulphation pattern of the CS chain is thought to be independently regulated from the rest of the CS chain such as the terminal non-reducing sugar[34,36]. The relevance of the different non-reducing terminal sugar residues of CS chains is still not clear but it could explain how CS chain elongation is controlled[36]. We were able to identify two non-reducing terminal sugars; GalNAc4S and GalNAc6S. We did not detect GalNAc4,6S in any of our subjects possibly because levels were too low for detection by FACE or that the terminal saccharide

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

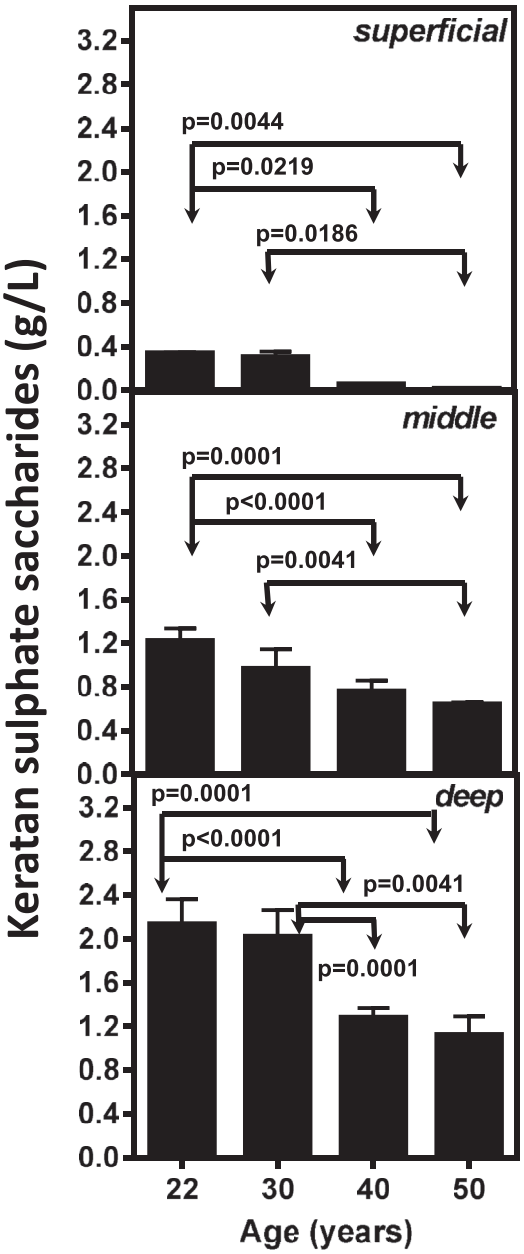

Fig. 4. The concentration of keratan sulphate (KS) saccharides (g/L) within the superficial, middle and deep zones of an age range (22, 30, 40 & 50 years) of human articular cartilage biopsies. The graph illustrates the mean concentration (g/L) ± 95% confidence limits (CL) for the eight biopsies. Zones with significantly different KS saccharide content when compared with those ages are indicated by arrows on the associated horizontal bars.

had already been catabolically removed into the synovial fluid. To our knowledge there have been no studies to investigate the functional implications of the different terminal structures on CS chains in cartilage.

KS, like CS, is an important component of aggrecan and together they provide cartilage with its resistance to physical stress and load[27]. The KS digestion products identified in our study concurred with previously published studies employing FACE[19,37]. The relative proportion of KS increased progressively through the depth of cartilage with increasing age. Using endo-β-galactosidase allows the identification of products from unsulphated regions of KS[21,22,37]. It was interesting to find unsulphated disaccharide

Table III
The calculated data for the keratan sulphate saccharides, as detected by FACE analysis. The values represent the mean concentration (g/L) and their 95% confidence intervals (CI; lower limit, upper limit) of eight biopsies taken from one area. Data are shown for each subject aged 22, 30, 40 and 50 years old. ND represents non detectable by FACE analysis

Keratan sulphate saccharides	Mean concentration (lower limit, upper limit) g/L											
Age (years)	22			30			40			50		
Zone	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep	Superficial	Middle	Deep
Non-reducing terminus	**0.091**	**0.051**	**0.087**	**0.045**	**0.045**	**0.070**	ND	**0.001**	**0.002**	ND	**0.001**	**0.002**
NeuAcα2,3Galβ1,4GlcNAc6S	(0.081,0.101)	(0.043,0.059)	(0.071,0.103)	(0.036,0.054)	(0.036,0.054)	(0.069,0.071)		(0.000,0.001)	(0.001,0.002)		(0.000,0.001)	(0.001,0.003)
Internal chain	**0.147**	**0.474**	**0.800**	**0.170**	**0.460**	**0.721**	ND	**0.512**	**0.870**	ND	**0.337**	**0.675**
Gal6Sβ1,4GlcNAc6S	(0.144,0.150)	(0.445,0.503)	(0.744,0.856)	(0.148,0.192)	(0.362,0.558)	(0.651,0.789)		(0.479,0.545)	(0.803,0.937)		(0.323,0.351)	(0.648,0.702)
Galβ1,4GlcNAc6S	**0.071**	**0.196**	**0.070**	**0.047**	**0.018**	**0.391**	**0.051**	**0.132**	**0.244**	ND	**0.134**	**0.232**
	(0.070,0.072)	(0.144,0.248)	(0.051,0.089)	(0.046,0.048)	(0.017,0.019)	(0.304,0.478)	(0.050,0.052)	(0.063,0.201)	(0.243,0.245)		(0.133,0.135)	(0.158,0.306)
Galβ1,2[fucα1,3]GlcNAc6S	ND	ND	ND	ND	ND	ND	ND	**0.101**	**0.130**	**0.010**	**0.161**	**0.211**
								(0.095,0.107)	(0.117,0.143)	(0.009,0.011)	(0.155,0.167)	(0.123,0.299)
GlcNAcβ1,3Gal	**0.021**	**0.180**	**0.241**	**0.015**	**0.151**	**0.29**	**0.003**	ND	ND	ND	ND	ND
	(0.020,0.022)	(0.176,0.184)	(0.232,0.248)	(0.011,0.019)	(0.112,0.190)	(0.213,0.361)	(0.001,0.005)					
GlcNAc6Sβ1,3Gal	**0.013**	**0.320**	**0.629**	**0.029**	**0.290**	**0.552**	**0.002**	**0.015**	**0.028**	**0.003**	**0.005**	**0.007**
	(0.012,0.014)	(0.278,0.362)	(0.545,0.713)	(0.026,0.032)	(0.263,0.317)	(0.500,0.656)	(0.001,0.003)	(0.007,0.023)	(0.013,0.043)	(0.002,0.004)	(0.004,0.006)	(0.006,0.008)

The mean concentration is in boldface.

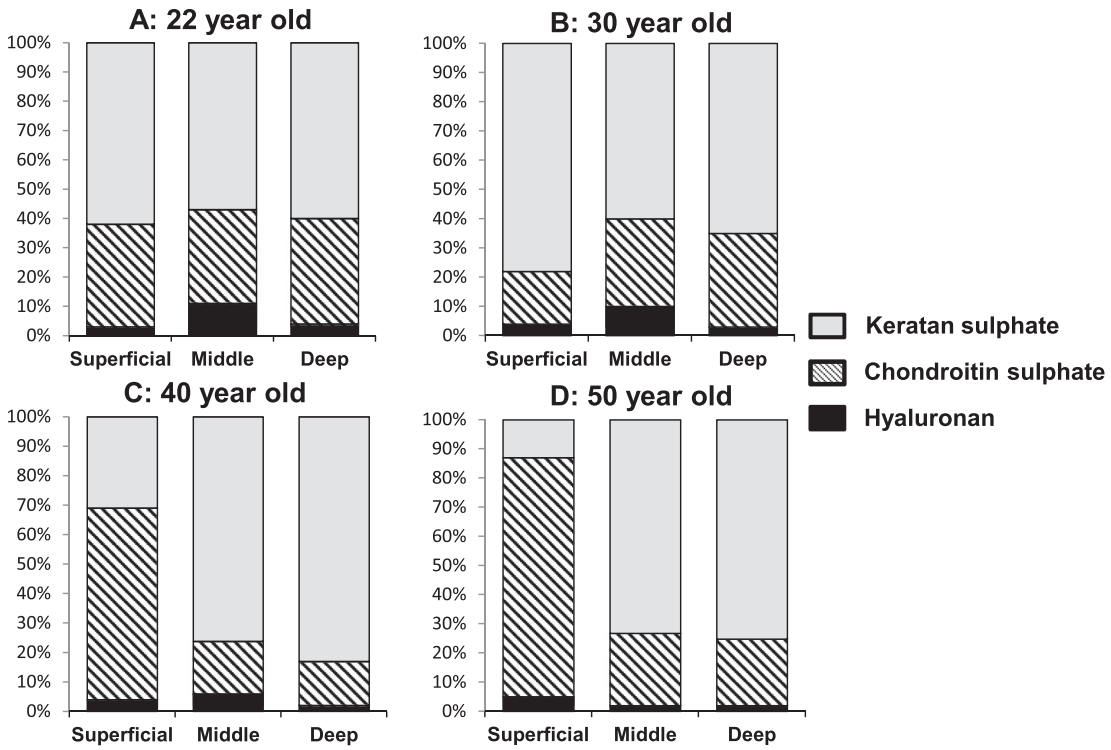

Fig. 5. The differences in the proportions of KS, HA and CS within the superficial, middle and deep zones of human cartilage biopsies from **A: 22 year old, B: 30 year old, C: 40 year old and D: 50 year old**. Each mean GAG concentration (g/L) for the eight biopsies, with its constituent disaccharide and monosaccharide components, is expressed as a total percentage of GAGs analysed by FACE.

(GlcNAcβ1,3Gal) in the 22 and 30 year old biopsies but not in the 40 and 50 year old biopsies. The sulphation levels of KS increased from around 50–80% with advancing age. For the more heavily sulphated KS, there appeared to be an inverse relationship between tissue depth and KS content. This would suggest that chondrocytes synthesise more sulphated GAGs when in conditions of hypoxia. One could speculate that in the middle and deep zones, the sulphation patterns of the KS components on proteoglycans influence their aggregation and dissociation.

All of our data largely agree with the previously published work by Elliott & Gardner[24] who assessed surface and deep cartilage by cellulose acetate electrophoresis. The use of the powerful and sensitive approach of FACE has enabled us to further build upon their work to provide detailed analysis of HA, CS and KS. From our study, we believe that we have shown that the absolute concentrations of GAG are just as important as their distribution. We are already using FACE to fully characterise native cartilage and tissue generated through cartilage regenerative cell therapy approaches. Ideally we could use FACE in combination with existing immunological methods as another useful outcome measure.

Contributions

Dr Kuiper was responsible for obtaining the funding for the study. She prepared the ethics paperwork for Trent Research Ethics Committee, UK in order to obtain the cartilage biopsies from the UK Human Tissue Bank. Dr Kuiper was responsible for the conception, study design and analysis of the data. Dr Kuiper drafted and then finally approved the article. Dr Sharma was responsible for the safe handling and recording of the biopsies. She performed the experiments and also analysed the data. She helped to draft the article.

Role of the funding source

The authors received a project grant from Arthritis Research UK.

Conflict of interest

The authors have no competing interests.

Acknowledgements

The authors would like to thank Arthritis Research UK (16181) for funding this work.

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 Oct 6;331(14):889–95.
2. Ding C, Jones G, Wluka AE, Cicuttini F. What can we learn about osteoarthritis by studying a healthy person against a person with early onset of disease? Curr Opin Rheumatol 2010 Sep;22(5):520–7.
3. Dewan AK, Gibson MA, Elisseeff JH, Trice ME. Evolution of autologous chondrocyte repair and comparison to other cartilage repair techniques. Biomed Res Int 2014;2014. 272481.
4. Buckwalter JA, Mankin HA. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 1998;47:477–86.
5. Coates EE, Fisher JP. Phenotypic variations in chondrocyte subpopulations and their response to *in vitro* culture and external stimuli. Ann Biomed Eng 2010 Nov;38(11):3371–88.
6. Quinn TM, Hunziker EB, Häuselmann HJ. Variation of cell and matrix morphologies in articular cartilage among locations in

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011

the adult human knee. Osteoarthritis Cartilage 2005 Aug;13(8):672–8.
7. Bayliss MT, Venn M, Maroudas A, Ali SY. Structure of proteoglycans from different layers of human articular cartilage. Biochem J 1983;209:387–400.
8. Hardingham TE, Fosang AJ. Proteoglycans – many forms and many functions. Faseb J 1992;6:861–70.
9. Vynios DH. Metabolism of cartilage proteoglycans in health and disease. Biomed Res Int 2014:2314–6133. 452315.
10. Bastow ER, Byers S, Golub SB, Clarkin CE, Pitsillides AA, Fosang AJ. Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci 2008;65:395–413.
11. Sasisekharan R, Raman R, Prabhakar V. Glycomics approach to structure-function relationships of glycosaminoglycans. Annu Rev Biomed Eng 2006;8:181–231.
12. Okolicsanyi RK, Griffiths LR, Haupt LM. Mesenchymal stem cells, neural lineage potential, heparan sulfate proteoglycans and the matrix. Dev Biol 2014 Apr 1;388(1):1–10.
13. Kramer KL. Specific sides to multifaceted glycosaminoglycans are observed in embryonic development. Semin Cell Dev Biol 2010 Aug;21(6):631–7.
14. Stockwell RA, Scott JE. Distribution of acid glycosaminoglycans in human articular cartilage. Nature 1967;215:1376–8.
15. Hayes AJ, Hughes CE, Caterson B. Antibodies and immunohistochemistry in extracellular matrix research. Methods 2008 May;45(1):10–21.
16. Bayliss MT, Osborne D, Woodhouse S, Davidson C. Sulfation of chondroitin sulfate in human articular cartilage. The effect of age, topographical position, and zone of cartilage on tissue composition. J Biol Chem 1999;274:15892–900.
17. Rogers BA, Murphy CL, Cannon SR, Briggs TW. Topographical variation in glycosaminoglycan content in human articular cartilage. J Bone Joint Surg Br 2006 Dec;88(12):1670–4.
18. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173–7.
19. Calabro A, Midura R, Wang A, West L, Plaas A, Hascall VC. Fluorophore-assisted carbohydrate electrophoresis (FACE) of glycosaminoglycans. Osteoarthritis Cart 2001;9(Suppl A): S16–22.
20. Karousou E, Asimakopoulou AP, Zafeiropoulou V, Viola M, Monti L, Rossi A, *et al.* Fast screening of glycosaminoglycan disaccharides by fluorophore-assisted carbohydrate electrophoresis (FACE): applications to biologic samples and pharmaceutical formulations. Methods Mol Biol 2015;1229: 143–59.
21. 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.
22. Wang QG, Hughes N, Cartmell SH, Kuiper NJ. The composition of hydrogels for cartilage tissue engineering can influence glycosaminoglycan profile. Eur Cell Mater 2010;Feb 26;19: 86–95.
23. Holmes MW, Bayliss MT, Muir H. Hyaluronic acid in human articular cartilage. Age-related changes in content and size. Biochem J 1988;250:435–41.
24. Elliott JR, Gardner DL. Changes with age in the glycosaminoglycans of human articular cartilage. Ann Rheumatic Dis 1979;38:371–7.
25. Plaas AH, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC. Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. J Biol Chem 1997;272:20603–10.
26. Nieduszynski IA, Huckerby TN, Dickenson JM, Brown GM, Tai GH, Morris HG, *et al.* There are 2 major types of skeletal keratan sulfates. Biochem J 1990;271:243–5.
27. Funderburgh JL. Keratan sulfate: structure, biosynthesis, and function. Glycobiology 2000 Oct;10(10):951–8.
28. Chen SS, Falcovitz YH, Schneiderman R, Maroudas A, Sah RL. Depth-dependent compressive properties of normal aged human femoral head articular cartilage: relationship to fixed charge density. Osteoarthritis Cartilage 2001;9:561–9.
29. Asari A, Miyauchi S, Kuriyama S, Machida A, Kohno K, Uchiyama Y. Localization of hyaluronic acid in human articular cartilage. J Histochem Cytochem 1994 Apr;42(4):513–22.
30. Zheng CH, Levenston ME. Fact versus artifact: avoiding erroneous estimates of sulfated glycosaminoglycan content using the dimethylmethylene blue colorimetric assay for tissue-engineered constructs. Eur Cell Mater 2015 Apr 19;29:224–36.
31. Fisher MB, Henning EA, Söegaard NB, Dodge GR, Steinberg DR, Mauck RL. Maximizing cartilage formation and integration via a trajectory-based tissue engineering approach. Biomaterials 2014 Feb;35(7):2140–8.
32. Khoshgoftar M, Wilson W, Ito K, van Donkelaar CC. Influence of the temporal deposition of extracellular matrix on the mechanical properties of tissue-engineered cartilage. Tissue Eng Part A 2014 May;20(9–10):1476–85.
33. Mikami T, Kitagawa H. Biosynthesis and function of chondroitin sulfate. Biochim Biophys Acta 2013 Oct;1830(10): 4719–33.
34. Lauder RM, Huckerby TN, Brown GM, Bayliss MT, Nieduszynski IA. Age-related changes in the sulphation of the chondroitin sulphate linkage region from human articular cartilage aggrecan. Biochem J 2001 Sep 1;358(Pt 2):523–8.
35. Calabro A, Hascall VC, Midura RJ. Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage. Glycobiology 2000;10: 283–93.
36. Little PJ, Ballingera ML, Burcha ML, Osmana N. Biosynthesis of natural and hyperelongated chondroitin sulfate glycosaminoglycans: new insights into an elusive process. Open Biochem J 2008;2:135–42.
37. 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;11:779–90.

Please cite this article in press as: Kuiper NJ, Sharma A, A detailed quantitative outcome measure of glycosaminoglycans in human articular cartilage for cell therapy and tissue engineering strategies, Osteoarthritis and Cartilage (2015), http://dx.doi.org/10.1016/j.joca.2015.07.011