View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Osteoarthritis and Cartilage 20 (2012) 396–404

Osteoarthritis and Cartilage

Is cartilage sGAG content related to early changes in cartilage disease? Implications for interpretation of dGEMRIC

J.J. Stubendorff †*, E. Lammentausta †‡, A. Struglics §, L. Lindberg †, D. Heinegård ||, L.E. Dahlberg †

† Joint and Soft Tissue Unit, Department of Clinical Sciences Malmö, Lund University, Department of Orthopaedics, Skåne University Hospital, Malmö, SE-20502 Malmö, Sweden
‡ Department of Diagnostic Radiology, Oulu University Hospital, POB 50, FI-90029 OYS, Oulu, Finland
§ Department of Orthopaedics, Clinical Sciences Lund, Lund University, Lund 221 84, Sweden
|| Department of Clinical Sciences Lund, BMC C12 Lund University, SE-22184 Lund, Sweden

ARTICLE INFO

Article history:
Received 7 September 2011
Accepted 17 January 2012

Keywords:
OA cartilage
dGEMRIC
sGAG content

SUMMARY

Objective: This study investigates sulphated glycosaminoglycans (sGAG) content changes in early osteoarthritis (OA), and whether contrast-enhanced magnetic resonance imaging (MRI) of cartilage *in vitro* may identify early event of OA pathology.
Method: Osteochondral plugs from patients with hip OA or femoral neck fracture (reference group) were collected and analysed by 1.5 T MRI with ΔR1 as a measure of cartilage contrast concentration. Cartilage hydration, contents of sGAG, cartilage oligomeric matrix protein (COMP), hydroxyproline, denatured collagen, and aggrecan $TEGE^{392}$ neoepitope were determined and histological grading was performed.
Results: sGAG content correlated to ΔR1, although no difference in either of these parameters was detectable between OA and reference cartilage at 4 h of contrast equilibration. In contrast, biochemical analysis of other cartilage matrix constituents showed distinct alterations typical for early cartilage degradation in OA cartilage and with clear evidence for increased aggrecan turnover.
Conclusion: In the present *in vitro* study, cartilage sGAG content could not distinguish between early OA cartilage and reference cartilage. Given, that delayed gadolinium enhanced MRI of cartilage (dGEMRIC) indicates early events in the pathogenesis of OA *in vivo*, our results from the *in vitro* studies imply other, additional factors than cartilage sGAG content, e.g., alterations in diffusion or increased supply of contrast agent in the diseased joint. Alternatively, an altered dGEMRIC reflects later stages of OA, when sGAG content decreases. Further investigations are warranted, to understand variations in sGAG content in pathology, an essential background for interpreting dGEMRIC measurements.
© 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Introduction

Detection of early events in osteoarthritis (OA) is of major interest in cartilage research. During developing cartilage damage, the role of extracellular matrix molecules has been investigated[1]. Sulphated glycosaminoglycans (sGAG), mainly situated on aggrecan, has been considered to play a central role in cartilage homeostasis, since its negative charge attracts water into cartilage, creating a high osmotic pressure. This is counteracted by the tensile strength of an intact collagen network[2,3], which is arranged by other cartilage matrix proteins such as cartilage oligomeric matrix protein (COMP)[4,5].

In pathology, the balance between synthesis and degradation of cartilage matrix constituents is altered[6]. The aggrecan derived ^{393}ARGS neoepitope (from aggrecanase cleavage at $TEGE^{392}$-↓^{393}ARGS)[7] has been proposed to reflect early pathology, seen as elevating ^{393}ARGS levels in the synovial fluid of OA and knee injured patients[8,9]. Increased serum COMP levels in patient groups with joint disease have been related to a more rapid progress of tissue destruction[10,11].

Delayed gadolinium enhanced magnetic resonance imaging of cartilage (dGEMRIC), introduced in 1996[12], is based on an inverse relationship between the cartilage distribution of the negatively charged contrast agent gadolinium-diethylene-triaminepentaacetic acid ($Gd\text{-}DTPA^{2-}$) and the fixed charged density, which is related to sGAG content[13]. *In vitro* studies show that the contrast medium distributes according to the Gibbs–Donnan equilibrium[14]. It has been suggested that the same phenomenon is valid also *in vivo*[15]. Early changes of OA are suggested to be accompanied with

* Address correspondence and reprint requests to: J.J. Stubendorff, Joint and Soft Tissue Unit, Department of Clinical Sciences Malmö, Lund University, Department of Orthopaedics, Skåne University Hospital, Malmö, SE-20502 Malmö, Sweden. Tel: 46-40-332450; Fax: 46-40-336200.
E-mail address: Johann.Stubendorff@med.lu.se (J.J. Stubendorff).

1063-4584/$ – see front matter © 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.joca.2012.01.015

a decrease in cartilage sGAG content and a number of clinical studies have been using dGEMRIC to study cartilage quality[16–18].

However, the mechanism is not clear how early changes of OA are accompanied by changes in cartilage sGAG content and there are conflicting data suggesting elevated[19–21], unchanged[22–24] and decreased[25,26] sGAG contents.

Therefore, we aimed with this study, to investigate: (1) how sGAG content reflects degradation in cartilage, (2) whether dGEMRIC can confirm changed sGAG *in vitro*, (3) and thereby demonstrate early OA pathology *in vitro*.

Materials and methods

Patients and cartilage samples

Femoral heads of patients with OA ($n = 8$, median age = 62 years, range: 51–68 years) and of patients with osteoporotic femoral neck fracture (reference cartilage, REF, $n = 8$, median age = 81.5 years, range: 78–89 years), due to low impact fall trauma, were collected at surgery and frozen at $-20°C$ tightly covered with Parafilm until further analysis. The study has been approved by the institutional review board.

Preparation for contrast-enhanced MRI

Femoral heads were thawed and cartilage bone plugs with a diameter of 6 mm were punched out. We harvested full thickness cartilage from the superior, habituell loaded part of the caput [Fig. 1(a)] with an intact surface as close as possible to the sites of advanced destruction [i.e., OA, Fig. 1(b)] and at corresponding regions with no sign of macroscopic damages (i.e., REF).

The plugs were arranged in a box with eight chambers and equilibrated in 15 ml phosphate buffered saline (PBS), pH 7.4 for 4 h at room temperature. Since the subchondral bone was not separated from the cartilage, the PBS medium had only access to the cartilage *via* three surfaces. The box was then placed in a 1.5 T system (Siemens Magnetom Sonata, Siemens Healthcare, Erlangen, Germany) with a dedicated knee coil, in a manner to allow measurement of eight samples at the same time (Fig. 2).

Contrast-enhanced MRI

For T_1 measurement, a single-slice inversion recovery spin echo sequence was used for both pre-contrast (TR = 3000 ms, TE = 15 ms, six TI's between 100 and 2800 ms, ETL = 11, matrix = 256 × 256, FOV = 143 × 143 mm, in-plane resolution 0.47 mm, slice thickness = 3 mm) and post-contrast (TR = 2000 ms, six TI's between 50 and 1600 ms, other parameters unchanged) time points. A single slice was localized to cover the central part of all the samples. The scan times were 9 min, and 6 min for pre-contrast and post-contrast T_1 measurements, respectively. After pre-contrast measurement, the samples were equilibrated in 1 mM contrast agent solution Magnevist, (Bayer/Germany) or Omniscan (GE Healthcare Oslo/Norway). Post-contrast T_1 measurements were performed immediately and 1, 2, 3 and 4 h after contrast agent injection. In the first setting including four OA and four REF samples, we used two adjacent biopsy specimens for the different contrast agents. In these samples, we measured T_1 additionally after 24 h of equilibration, which did not show differences compared to 4 h of equilibration (data not shown). In the other setting (also four of each samples), we used the identical sample for first the charged (i.e., Magnevist) and then the non-charged (i.e., Omniscan) contrast agent. In between the different sets of contrast, we washed out the charged agent thoroughly by changing the surrounding PBS 7 times over a time period of 24 h. T_1 measurements at 4 h after contrast agent administration did not show differences, when the first setting was compared to the second setting.

T_1 calculations and segmenting the regions of interest were done using MATLAB® (Mathworks Inc., Natick, MA) and MRI-mapper (Massachusetts Institute of Technology and Beth Israel Deaconess Medical Centre, Boston, MA, USA). Full-thickness region of interest (ROI) was manually segmented into each cartilage piece [Fig. 2(a/b)]. To avoid partial volume artefacts, the edges of the samples were excluded in the radial direction.

To demonstrate the gadolinium (Gd) concentration in the cartilage at different time points, the results for each sample are shown as $\Delta R_1 = (1/T_{1Gd} - 1/T_{1pre})$, where T_{1Gd} is the T_1 value at a certain time point after contrast agent injection and T_{1pre} is the T_1 value before Gd-DTPA^{2-} injection. T_{1Gd} usually depicts the dGEMRIC index in clinical *in vivo* studies.

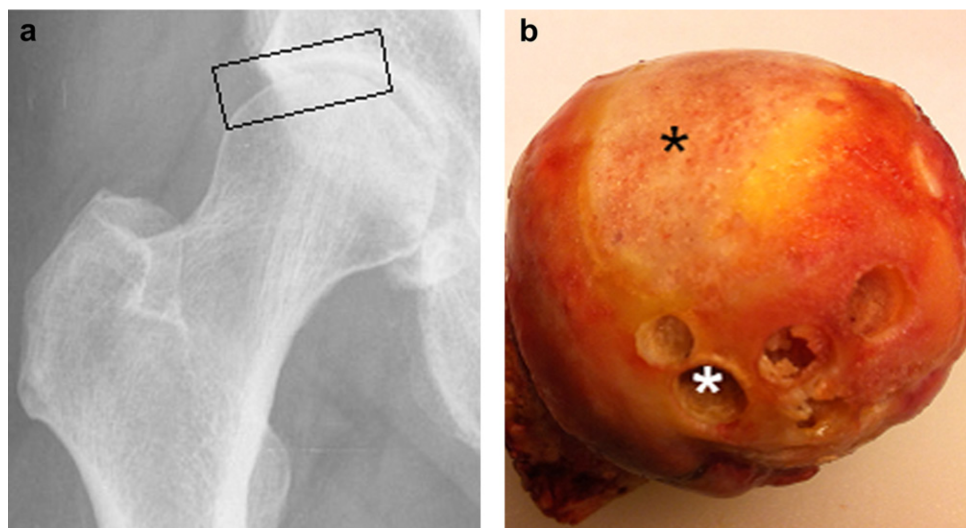

Fig. 1. (a) Anterior-posterior X-ray of a hip joint. The rectangle illustrates the region of sample harvesting in both OA and REF cartilages. (b) Representative cranial view photograph of an OA caput femoris. Black asterisk: lack of cartilage due to OA. White asterisk: full thickness sample as close as possible to degradation.

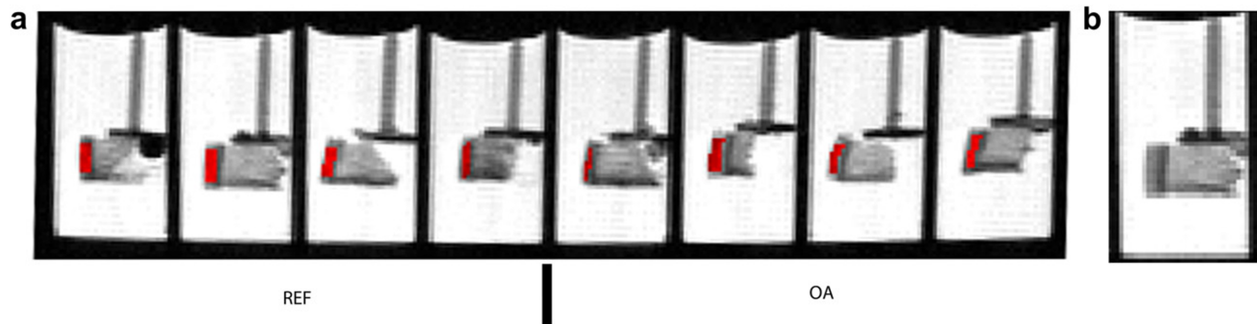

Fig. 2. (a and b): Representative ROI (red areas) illustrated on four REF (on the left) and four OA cartilage samples (on the right) at 4 h after addition of Magnevist. The specimens were hold on the subchondral bone in plastic devices, "hanging" in the surrounding PBS medium (15 ml in every box) allowing the contrast agent penetrating from three sides into the cartilage. The pins of the sample holding devices are attached at the centre of the sample clamp, and indicate that the samples are satisfactorily arranged in the same orientation as the MRI scan is performed. (b) Segmentation of representative sample: the subchondral bone appears as a black stripe between the cartilage and the trabecular bone. To avoid partial volume artefacts, the edges of the samples were excluded in the radial direction.

Preparation for biochemical analyses

After MRI measurements, the PBS media were saved and frozen at −20°C until further analysis. The cartilage pieces were cut from the subchondral bone and divided in two pieces, one for extraction and quantification of extracellular matrix (ECM) molecules, and the other for histology. For the samples aimed for protein quantification, we obtained the wet weight and dry weight, after freeze-drying. The water content was calculated [((wet weight − dry weight)/wet weight) × 100]. The samples for histology were frozen until further analyses.

Protein extraction

The cartilage samples were cut into small pieces and extracted with 4 M Gu–HCl containing protease-inhibitor cocktail for 24 h at 4°C as previously[5]. After centrifugation, the pellets were frozen at −20°C until further analysis. The supernatants were used to quantify sGAG, COMP, and aggrecan TEGE fragments.

Protein quantification in cartilage extracts

In OA and REF cartilage extracts, sGAG contents were quantified by Alcian Blue measurements according to the manufacture (Wieslab, Lund)[27]. COMP was quantified with an inhibition ELISA using human recombinant COMP as coat and an anti bovine COMP antibody[28]. The total amount of extracted protein was determined by BRADFORD-assay[29]. All biochemical variables were related to tissue wet weight, since we interpret cartilage water content as a functional compartment.

Protein quantification in PBS medium

In order to enrich the protein concentration, in the 15 ml PBS medium (from MRI measure), two 3 ml aliquots were precipitated twice with 10 volumes of 96% ethanol, containing 50 mM NaAc at 4°C over night. The precipitates were recovered by centrifugation and either dissolved in 100 μl COMP-ELISA buffer (PBS, pH 7.4, containing 0.8% SDS)[28] or dissolved in 100 μl 4 M Gu–HCl for sGAG quantification.

Quantification of TEGE^{392}

Gu–HCl extracts were prepared and used for the quantification of aggrecanase generated aggrecan neoepitope TEGE^{392}. Samples were deglycosylated and prepared as described[30,31], and applied on 4–12% Bis–Tris SDS-PAGE mini gels (Invitrogen). An anti-NITEGE antibody (i.e., sera[32]) was used to detect the aggrecan neoepitope TEGE^{392} and to quantify its relative abundance by Western blot using a luminescence image analyzer (Fujifilm LAS 1000)[30].

Aggrecan amino acid numbers in this paper are based on full length human sequences starting with the N-terminal ^{1}MTTL (NCBI accession nr P16112).

Collagen analysis

The residual pellets after Guanidine–HCl extraction were used to determine collagen content by measurement of hydroxyproline by the Stegmann–Stalder colourimetric procedure[33]. The percentage of extractable collagen (i.e., denatured collagen) was analysed as described[34].

Histology

Corresponding cartilage pieces were thawed and embedded in Paraffin. Sections were cut (7 μm), stained with Safranin-O and Fast Green[35] and graded according to Mankin[36] independently by three investigators. Mean values are presented.

Statistical procedures

Statistical calculations were performed with SPSS 17.0 for Windows. Group differences were determined by Mann–Whitney *U* test for group comparisons. To test for relationships between parameters, Spearman's rank (r_s) correlation tests were performed. One sample of the OA group (sample #48) has been excluded from statistical analyses (see Results).

Reproducibility/reliability

For biochemical variables (sGAG, COMP, TEGE, hydroxyproline, denatured collagen), established methods have been described elsewhere[27–31,33,34]. For each variable, samples were analysed in duplicates, and the means were used for further evaluations.

MRI measurements: the measurement chamber was never removed from the coil between the T1 measurements. Therefore, the ROIs could be copied from the first time point to all the other time points, and the possible error due to sample repositioning was eliminated. The differentiating of cartilage from the surrounding materials (PBS or Gd solution on three sides, subchondral bone on one side) was easy. Even though the specimens were quite small and the resolution was low, we are confident, that the ROIs

represent cartilage [Fig. 2(a/b)]. For *in vivo* studies, dGEMRIC has shown to have good reproducibility, provided the same ROI is chosen[37]. The current setup mimicks the *in vivo* measurements, and because the repositioning error is eliminated, the reproducibility of this setup can be considered to be equal or better than that of *in vivo* setup.

Results

Contrast-enhanced MRI

The distribution of Magnevist into OA and REF cartilage was fastest directly after the addition of the contrast agent and reached equilibrium after 3–4 h (Fig. 3). ΔR1 at 4 h did not show differences between OA and REF cartilages ($P = 0.798$).

The non-charged contrast agent Omniscan entered the cartilage at a higher rate compared to the charged Magnevist (Fig. 3). As for Magnevist there were no differences in Δ*R*1 between OA and REF cartilage at 4 h ($P = 0.721$). However, ΔR1 at 4 h in the non-charged Omniscan samples were higher compared to the charged Magnevist ($P < 0.001$, Fig. 3), indicating that more contrast agent had entered the cartilage.

Biochemical analyses of cartilage

OA samples had significant higher water contents than REF samples [median OA 74.0%, REF 66.9%, $P = 0.006$, Fig. 4(a)].

There were no differences in sGAG concentrations and amount of extractable proteins between OA and REF samples [Fig. 4(b), data not shown]. In contrast, COMP concentration was higher in OA- compared to REF-cartilage [$P = 0.006$, Fig. 4(c)]. One sample of the OA group (sample #48) contained almost no detectable sGAG and COMP, but released 98% of sGAG and 64% of COMP into the PBS medium during equilibration. It differed histologically from the others, by having deep cleavages in the matrix, no staining for proteoglycans in the deep layer and irregular staining for the collagen network [Fig. 5(d)], most likely representing much more advanced stages of OA. Therefore, sample #48 was excluded from the statistical data presented. The other cartilage samples showed a low release (sGAG release in OA samples 1.28% of the total sGAG content vs REF samples 0.44%, $P = 0.014$; COMP in OA samples 1.19% vs REF samples 0.96%, not significant).

The TEGE[392] epitope concentration, reflecting aggrecan fragmentation, was variable in the OA cartilage and was not different from the REF samples [$P = 0.152$, Fig. 4(d)].

The cartilage hydroxyproline content was lower in OA (9.44 μg/mg ww) than in REF samples (11.79 μg/mg ww) [$P < 0.001$, Fig. 4(e)] and in accordance the relative amount of denatured collagen was higher in OA than REF samples [9.39% vs 2.32%, $P = 0.009$, Fig. 4(f)].

Correlation analyses

We chose the hydration of the cartilage as a general indicator of cartilage quality[25,38]. The water content was negatively correlated with the collagen content ($r = -0.843$, $P < 0.001$) and positively correlated with the fraction of denatured collagen ($r = 0.512$, $P = 0.043$).

The relative abundance of the TEGE[392] epitope correlated to the water content ($r = 0.754$, $P = 0.001$), and also negatively to the hydroxyproline content ($r = -0.638$, $P = 0.008$). There was no correlation between cartilage sGAG or COMP concentration and water content [Fig. 5(a, b)].

sGAG content and histology

Histological grading according to Mankin[36] revealed that the OA samples (excluding sample #48) were more destroyed than the REF samples (median Mankin score 3.25 vs 1.25, $P < 0.001$). OA and REF sample pairs with similar sGAG and water contents (i.e., OA #41 – REF #38 and OA #47 – REF #45), and OA and REF pairs with similar sGAG- but different water contents (i.e., OA #36 – REF #44 and OA #35 – REF #39) were compared histologically [Fig. 5(c)]. Cell hypertrophy, hypercellularity and rough surfaces in the OA samples indicated degradation, even though the sGAG content was similar compared to REF cartilage [Fig. 5(a, c)].

sGAG content and dGEMRIC

After 4 h incubation with Magnevist the ΔR1 correlated to the sGAG content ($r = -0.618$, $P = 0.014$, Fig. 6), whereas incubation with Omniscan did not ($r = 0.246$, $P = 0.376$).

Discussion

In the present study, we characterised and compared OA hip cartilage with osteoporotic femoral neck fracture cartilage (reference) with the hypothesis that sGAG content in OA cartilage is decreased, reflecting early degradation[2,25,26,39,40]. We quantified sGAG with biochemical (Alcian Blue) and contrast-enhanced MRI (dGEMRIC) methods, prior to the analysis of cartilage composition with different biochemical tools. Although the number of samples was low, we observed significant differences in most of the biochemical parameters indicating different grades of cartilage destruction. As a notable exception, we were not able to detect differences in cartilage sGAG content.

Grushko *et al.* [25] showed that the sGAG content in normal hip cartilage is quite homogenous in the superior, anterior and posterior part of the femoral head, and the variation in sGAG and water content for patients in the corresponding age to the presently studied OA (62 years) and REF patients (81 years) are not pronounced. When diseased, significant increase of water content in age- and site-matched OA samples (hip fracture 70% up to OA 75%) has been reported[25]. In analogy, we suggest that differences in water content here observed (ca. 67% in REF cartilage vs 74% in OA) are not due to differences in age or localisation, but are related to

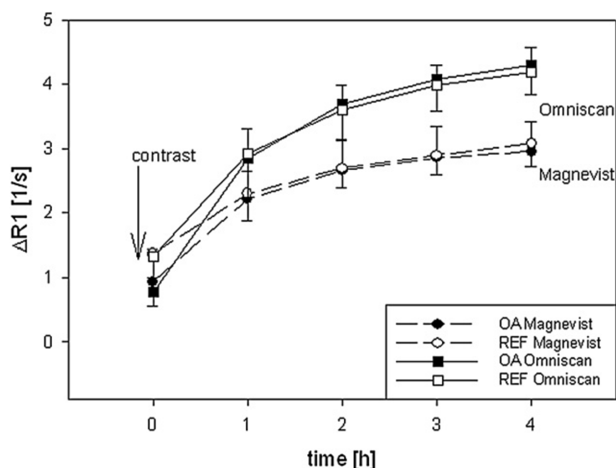

Fig. 3. Dynamics of the uptake of Magnevist and Omniscan. Mean-ΔR1 after addition of 1 mM contrast agent. Boxes indicate the use of Omniscan, circles the use of Magnevist. White symbols indicate REF cartilage ($n = 8$), black symbols indicate OA cartilage ($n = 7$). The arrow indicates the time point for addition of contrast agent. Error bars = 95% confidence interval.

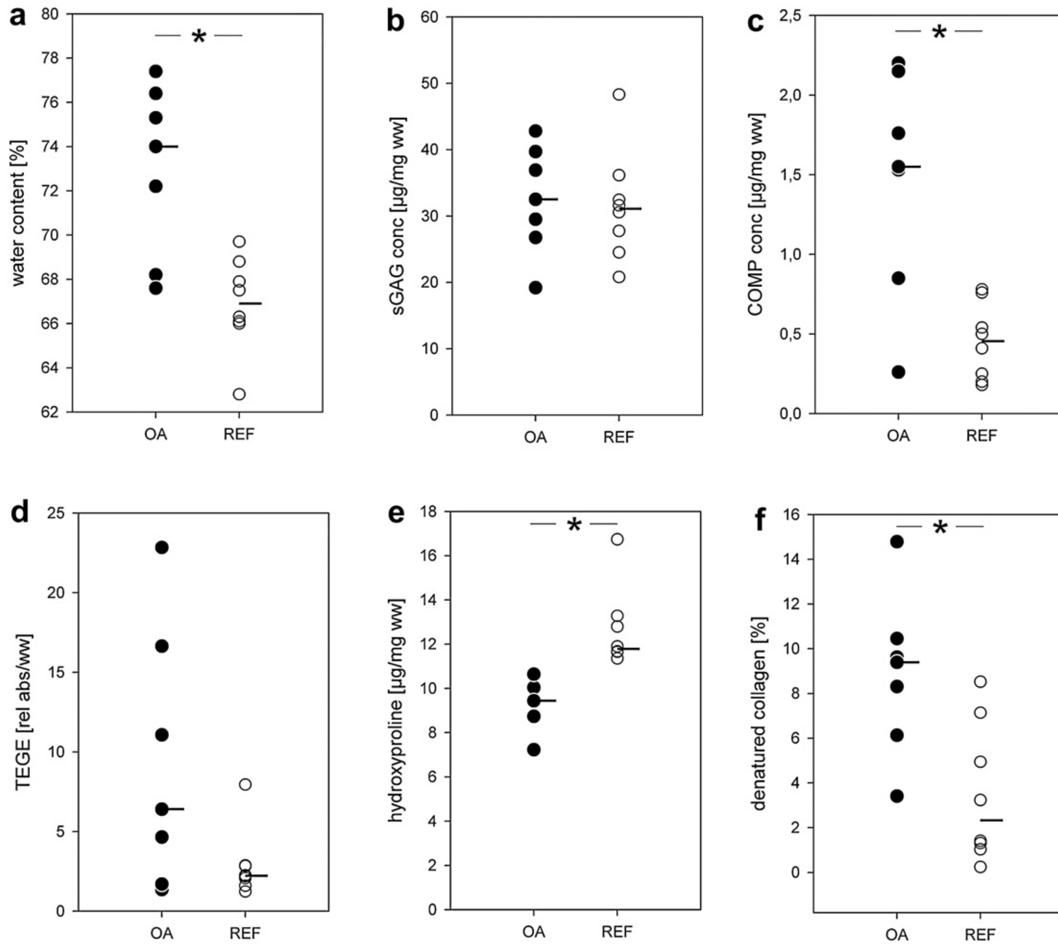

Fig. 4. Comparison of biochemical parameters (a: water, b: sGAG, c: COMP, d: TEGE, e: hydroxyproline, f: denatured collagen) between OA ($n = 7$) and REF ($n = 8$) cartilage. Black circles indicate OA cartilage, white circles indicate REF cartilage, and the line indicates the median. Individual circles represent mean values of duplicate measurements. * = Statistical significance ($P < 0.05$).

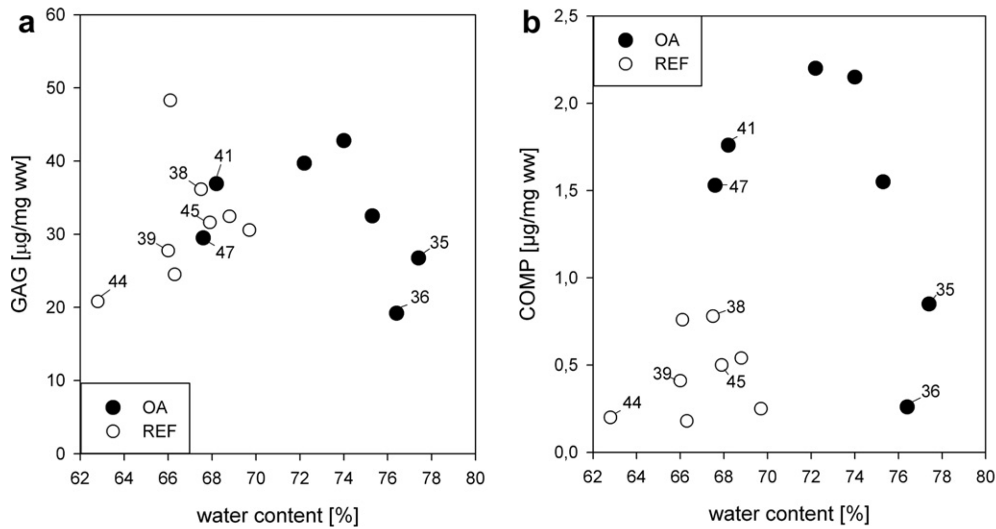

Fig. 5. (a) Relationship between water content and sGAG content. The numbers indicate samples which are compared histologically (in c). (b) Relationship between cartilage COMP content and water content. (c) Histological comparison of OA and REF cartilage with Safranin-O/Fast Green stained histological sections. The numbers correspond to the samples, chosen in Fig. 5a/b and the line corresponds to 100 pixels in the photo image, providing similar magnification in all sections (50×). (d) Sample #48, which released the protein content into PBS medium.

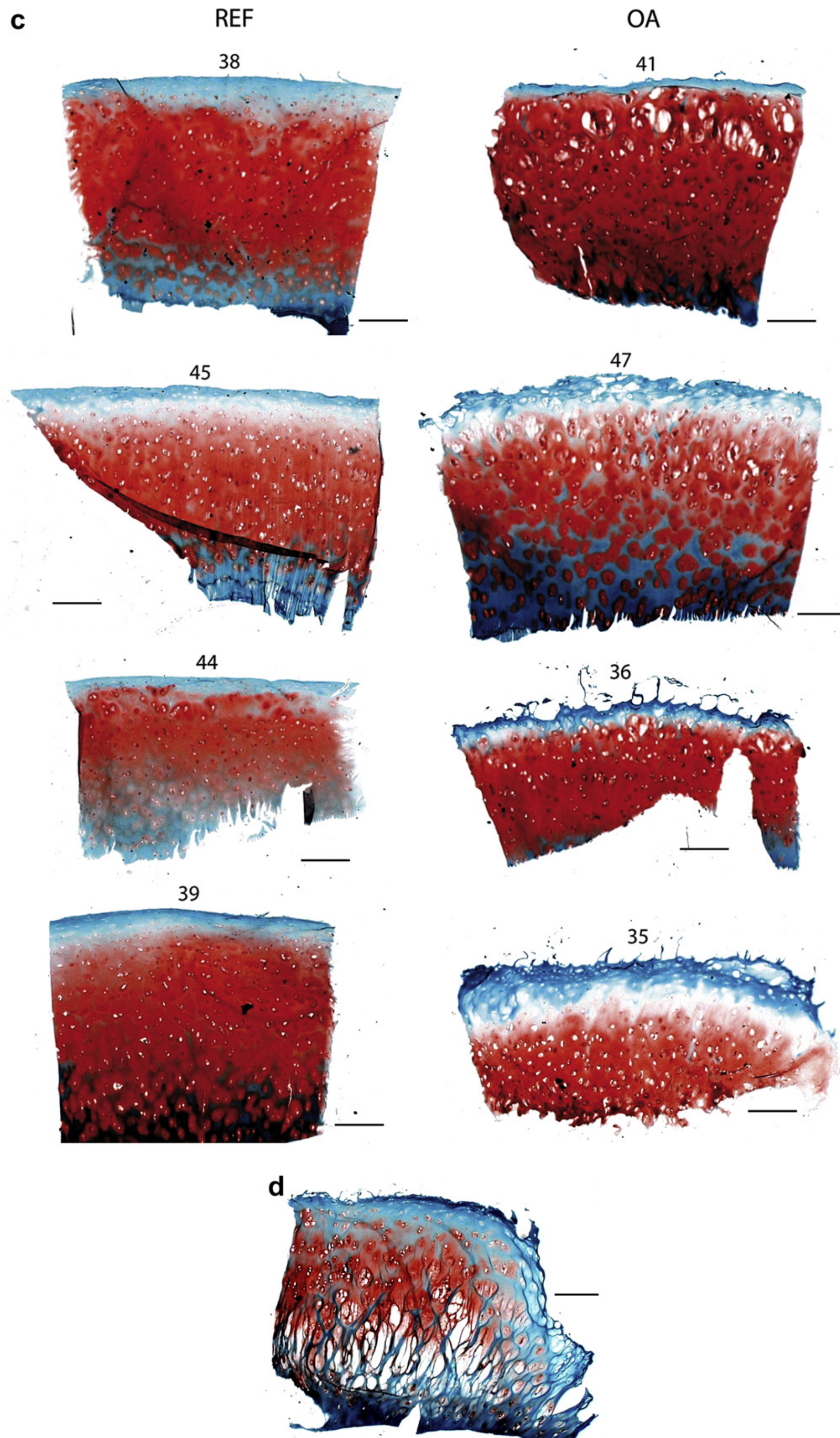

Fig. 5. (*continued*).

cartilage pathology. We interpreted the water content *in vitro* as a function of an impaired strength of the collagen network, which allows an increased water inflow and cartilage oedema[2,3].

For interpretation of dGEMRIC, sGAG *content* is of major interest in opposite to actual sGAG *synthesis*. Fibrillation of cartilage and occurrence of hypertrophic chondrocytes seem to be of importance, when monitoring sGAG content. Many studies report on a sGAG-decrease in fibrillated cartilage[25,26,39,40], whereas other studies report on a sGAG elevation in cartilage with hypertrophic chondrocytes[19,21].

In the present study, we had cartilage samples with visually intact surface without fibrillation (not including sample #48), which we consider being at early stages of degradation. The histology of two OA samples with normal hydration (meaning similar hydration compared to REF cartilage) and intact surface showed the occurrence of hypertrophic chondrocytes, possibly reflecting increased turnover of the ECM proteins [e.g., Fig. 5(c), samples #41 and #47], indicated also by an increased COMP content [Fig. 5(b)][20]. In contrast, OA samples with increased hydration, showed histological irregularities of the surface, no hypertrophic chondrocytes and decreasing COMP contents [Fig. 5(b)], probably reflecting stages before fibrillation and failing repair capacity [e.g., Fig. 5(c) sample #35 and #36]. In the highly fibrillated sample #48, we observed pronounced release of ECM molecules into the surrounding bath which may lead to underestimation of the actual cartilage sGAG content, at least in the experimental setting. Even the non-fibrillated OA samples released about three times more sGAG compared to the REF samples, though at low level, which does probably not significantly influence the sGAG cartilage content. In accordance to other studies, reporting elevated or no changes in sGAG content, we interpret our results as compensatory synthesis secondary to an increased release of sGAG from the ECM. In support it has been shown that the sGAG turnover is fast[24,41,42], indicating capacity of chondrocytes to counteract degenerative processes.

The data presented are in agreement with our previous data on early changes in knee OA showing no or minimal changes in the sGAG content, but major alterations in the content of extracellular matrix proteins[20].

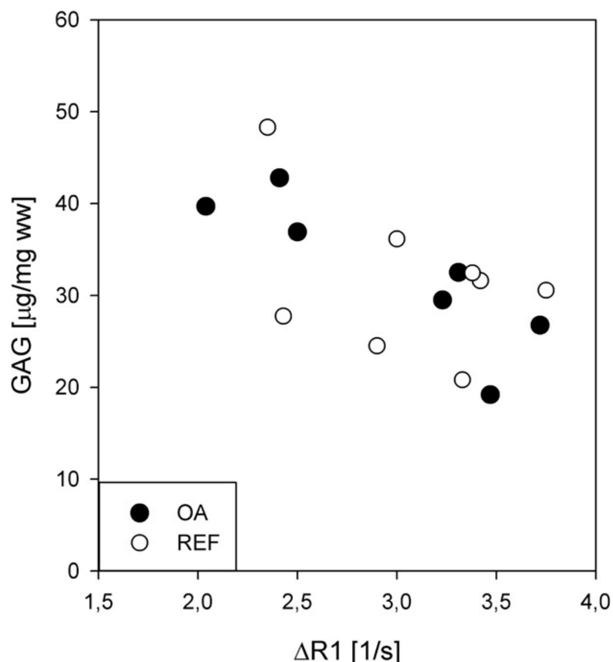

Fig. 6. Relationship between sGAG and ΔR1 after 4 h incubation with the contrast medium Magnevist.

In accordance to earlier studies[3,43] we found a relationship between increased hydration and increased amount of denatured collagen, reflected by the pool of collagen/hydroxyproline extractable with chymotrypsin[34]. We also found that the amount of total hydroxyproline was decreased in the OA cartilage and correlated to the water content. We conclude that in later stages of OA, indicated by increasing hydration, collagen fibres and molecules decorating the collagen fibril surface might become damaged by early degradative processes in combination with osmotic and biomechanic stress. The consequence is a looser collagen network and increase in hydration. Damaged collagen will be proteolysed and may be released out of the cartilage, which would explain the decrease of the total hydroxyproline. Another explanation is cartilage oedema with no compensatory increased collagen production.

Aggrecanases, key enzymes in early events of cartilage degradation, cleave aggrecan at TEGE$^{392}\downarrow^{393}$ARGS[7,44–46]. In OA the C-terminal aggrecan neoepitope TEGE^{392} at least initially remains in the ECM as a G1-TEGE fragment where the G1-domain is bound to hyaluronic acid *via* the link protein. We interpreted an increased abundance of TEGE^{392} as the accumulation of end products of degenerative turnover due to increased activity of aggrecanases in OA [Fig. 4(d)], reflecting cartilage degradation. Significant correlation between the abundance of TEGE^{392} and the water content and hydroxyproline content supports this hypothesis.

We found a significant increase in COMP content in the OA samples [Fig. 4(c)], reflecting changes in the cartilage turnover. According to its attributed role in collagen fibre organisation[4], its increased levels in serum samples of RA and OA patients[10,11], and in analogy to findings by Lorenzo *et al.*[20], we interpreted the increased COMP contents in our studies as increased synthesis secondary to repair attempts of the cartilage to degeneration.

The lower uptake of Magnevist compared to Omniscan (Fig. 3) was interpreted as depending on the charge. In agreement to Bashir *et al.*[12,15] we found a correlation between cartilage sGAG content and ΔR1-Magnevist (Fig. 6), and no correlation to ΔR1-Omniscan, supporting the principles of dGEMRIC. We found, however, in accordance with our biochemical results, no differences in ΔR1 between OA and REF cartilage (Fig. 3). In this respect, we were not able to confirm clinical dGEMRIC studies with intravenous injections of the contrast agent, which suggest a relationship between alterations of T1/ΔR1 and the future development of OA[16–18].

The Gibbs–Donnan equilibrium postulates equilibrium, requiring a steady state of influx and elimination. In contrast to *in vitro* studies, the human body is not a closed systems, since fast renal elimination and influx into different body compartments occur at the same time (e.g., fatfree-tissue[47], joint capsle and cartilage may be with different properties of contrast agent distribution[48]). Thus it might be difficult, to apply the Gibbs–Donnan equilibrium *in vivo* correctly and differences in dGEMRIC between diseased and healthy cartilage may therefore also be explained by other factors.

We suggest diffusion as one possible factor, which is dependent (1) on the supply of i.e., contrast agent, which might be increased in diseased joints by increased circulation due to inflammation, and (2) on composition of the cartilage matrix (water content, integrity of collagen network), which, when diseased, may allow a faster contrast agent distribution[49].

Presumed, the Donnan-theory is applicable *in vivo* and dGEMRIC is dependent on cartilage sGAG content only, our results would indicate, that dGEMRIC depicts individuals with later stages of OA, when cartilage is probably fibrillated already, accompanied by increased sGAG release and failing compensative sGAG synthesis. Changes of dGEMRIC index have also been related to level of physical activity in asymptomatic "healthy" volunteers, where the contribution of diffusion is unclear, but might be more important

than charge, since those subjects are assumed, to have increased cartilage sGAG content, which may alter diffusion. However, present *in vitro* results showed differences between the uptake of charged and non-charged contrast agent, indicating that the charge (of cartilage and contrast agent) does play a role. This implies, that differences of dGEMRIC *in vivo* may not be explained only by diffusion either. Using contrast enhanced MRI and comparing charged and non charged contrast agent *in vivo* may elucidate at what stage in OA cartilage sGAG content resp diffusion influence the dGEMRIC- index. In this respect, comparison can also be made between OA and healthy cartilage.

Study limitations: (1) As described above, differences in age between OA and REF group might affect the results, although our belief is that the differences seen between OA and REF groups can not be explained entirely by the differences in age, since Grushko *et al.* showed, that the sGAG content in normal hip cartilage for patients in the corresponding age to the presently studied OA and REF patients are not pronounced[25]. (2) In the present study we investigated only eight samples/individuals in each group. For most of the variables the differences between groups were large enough for statistical significance, although a larger sample size might change the non-significant data. (3) The resolution of contrast-enhanced MRI at 1.5 T is low, and small changes of ΔR1 after 4 h might not be detected. Although, we chose 1.5 T MRI, to keep the condition as similar as possible to the clinical dGEMRIC (1.5 T), since differences in relaxation times in cartilage have been reported, depending on field strength[50]. (4) We can not take into account possible spatial differences in sGAG concentration, since we analysed bulk cartilage only.

Summarised, we report similar sGAG level in cartilage samples from hip OA patients and patients with femoral neck fracture (reference samples), even though a number of biochemical parameters indicated pronounced differences typical for changes in early OA. sGAG content probably remains quite constant in early OA secondary to compensative sGAG synthesis.

We conclude that the measurement of cartilage sGAG content, measured *in vitro* by Alcian Blue precipitation or contrast-enhanced MRI is not a sensitive/specific method to detect early cartilage degeneration. In contrast, cartilage water content and COMP content distinguish early OA from histological non-arthritic reference cartilage. Given, that dGEMRIC indicates early events in the pathogenesis of OA *in vivo*, our results from the *in vitro* studies imply that in addition to cartilage sGAG content, other factors may also play a role, such as alterations in diffusion or increased supply of contrast agent in the diseased joint. Alternatively, dGEMRIC reflects later stages of OA, when sGAG content decreases. Further investigations need to be done to relate to, and understand the variations in sGAG content and other factors by pathology, which is mandatory for interpreting *in vivo* dGEMRIC measurements. Combination of biomarkers and cartilage imaging might aid in better understanding of early events in the development of OA.

Role of the funding source

Supported by grants from the Swedish Research Council, King Gustaf V's 80-year Fund, Swedish National Center for Research in Sports, The Herman Järnhardt Foundation, The Alfred Österlund Foundation and The Skåne University Hospital Funds.

Author contributions

Johann Stubendorff: Conception and design, sample collection, dGEMRIC measurements, COMP and TEGE quantification, statistical analyses, manuscript preparation. Support in protein extraction, sGAG, protein and hydroxyproline quantification, histology.

Eveliina Lammentausta: dGEMRIC measurements. Corresponding section in manuscript material and methods, manuscript revision.

André Struglics: Support with TEGE quantification, manuscript revision.

Lisbeth Lindberg: Sample storing, protein extraction; sGAG, protein and hydroxyproline quantification; histology.

Dick Heinegård: Support with COMP quantification, manuscript revision.

Leif Dahlberg: Conception and design. Manuscript revision.

Conflict of interest

There are no competing interests.

Acknowledgements

Carl Siversson and Jonas Svensson for the help to establish the *ex vivo* dGEMRIC system. Maria Hansson for technical support with TEGE quantification, Mette Lindell for technical support with COMP quantification.

References

1. Heinegard D. Proteoglycans and more – from molecules to biology. Int J Exp Pathol 2009;90:575–86.
2. Maroudas A, Venn M. Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. Ann Rheum Dis 1977;36:399–406.
3. Maroudas AI. Balance between swelling pressure and collagen tension in normal and degenerate cartilage. Nature 1976;260: 808–9.
4. Halasz K, Kassner A, Morgelin M, Heinegard D. COMP acts as a catalyst in collagen fibrillogenesis. J Biol Chem 2007;282: 31166–73.
5. Rosenberg K, Olsson H, Morgelin M, Heinegard D. Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. J Biol Chem 1998;273: 20397–403.
6. Heathfield TF, Onnerfjord P, Dahlberg L, Heinegard D. Cleavage of fibromodulin in cartilage explants involves removal of the *N*-terminal tyrosine sulfate-rich region by proteolysis at a site that is sensitive to matrix metalloproteinase-13. J Biol Chem 2004;279:6286–95.
7. Fosang AJ, Rogerson FM, East CJ, Stanton H. ADAMTS-5: the story so far. Eur Cell Mater 2008;15:11–26.
8. Larsson S, Lohmander LS, Struglics A. Synovial fluid level of aggrecan ARGS fragments is a more sensitive marker of joint disease than glycosaminoglycan or aggrecan levels: a cross-sectional study. Arthritis Res Ther 2009;11. R92.
9. Struglics A, Larsson S, Pratta MA, Kumar S, Lark MW, Lohmander LS. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloproteinase-generated aggrecan fragments. Osteoarthritis Cartilage 2006;14:101–13.
10. Mansson B, Carey D, Alini M, Ionescu M, Rosenberg LC, Poole AR, *et al.* Cartilage and bone metabolism in rheumatoid arthritis. Differences between rapid and slow progression of disease identified by serum markers of cartilage metabolism. J Clin Invest 1995;95:1071–7.
11. Sharif M, Saxne T, Shepstone L, Kirwan JR, Elson CJ, Heinegard D, *et al.* Relationship between serum cartilage oligomeric matrix protein levels and disease progression in osteoarthritis of the knee joint. Br J Rheumatol 1995;34:306–10.
12. Bashir A, Gray ML, Burstein D. Gd-DTPA2- as a measure of cartilage degradation. Magn Reson Med 1996;36:665–73.

13. Maroudas A, Muir H, Wingham J. The correlation of fixed negative charge with glycosaminoglycan content of human articular cartilage. Biochim Biophys Acta 1969;177:492–500.
14. Maroudas A. Physicochemical properties of cartilage in the light of ion exchange theory. Biophys J 1968;8:575–95.
15. Bashir A, Gray ML, Hartke J, Burstein D. Nondestructive imaging of human cartilage glycosaminoglycan concentration by MRI. Magn Reson Med 1999;41:857–65.
16. Ericsson YB, Tjornstrand J, Tiderius CJ, Dahlberg LE. Relationship between cartilage glycosaminoglycan content (assessed with dGEMRIC) and OA risk factors in meniscectomized patients. Osteoarthritis Cartilage 2009;17:565–70.
17. Owman H, Tiderius CJ, Neuman P, Nyquist F, Dahlberg LE. Association between findings on delayed gadolinium-enhanced magnetic resonance imaging of cartilage and future knee osteoarthritis. Arthritis Rheum 2008;58:1727–30.
18. Tiderius CJ, Olsson LE, Leander P, Ekberg O, Dahlberg L. Delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) in early knee osteoarthritis. Magn Reson Med 2003;49:488–92.
19. Adams ME, Brandt KD. Hypertrophic repair of canine articular cartilage in osteoarthritis after anterior cruciate ligament transection. J Rheumatol 1991;18:428–35.
20. Lorenzo P, Bayliss MT, Heinegard D. Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis. Matrix Biol 2004;23:381–91.
21. Matyas JR, Ehlers PF, Huang D, Adams ME. The early molecular natural history of experimental osteoarthritis. I. Progressive discoordinate expression of aggrecan and type II procollagen messenger RNA in the articular cartilage of adult animals. Arthritis Rheum 1999;42:993–1002.
22. Lorenz H, Wenz W, Ivancic M, Steck E, Richter W. Early and stable upregulation of collagen type II, collagen type I and YKL40 expression levels in cartilage during early experimental osteoarthritis occurs independent of joint location and histological grading. Arthritis Res Ther 2005;7:R156–65.
23. Mankin HJ, Lippiello L. The glycosaminoglycans of normal and arthritic cartilage. J Clin Invest 1971;50:1712–9.
24. Maroudas A. Glycosaminoglycan turn-over in articular cartilage. Philos Trans R Soc Lond B Biol Sci 1975;271:293–313.
25. Grushko G, Schneiderman R, Maroudas A. Some biochemical and biophysical parameters for the study of the pathogenesis of osteoarthritis: a comparison between the processes of ageing and degeneration in human hip cartilage. Connect Tissue Res 1989;19:149–76.
26. Yagi R, McBurney D, Laverty D, Weiner S, Horton Jr WE. Intrajoint comparisons of gene expression patterns in human osteoarthritis suggest a change in chondrocyte phenotype. J Orthop Res 2005;23:1128–38.
27. Karlsson M, Bjornsson S. Quantitation of proteoglycans in biological fluids using Alcian blue. Methods Mol Biol 2001;171: 159–73.
28. Saxne T, Heinegard D. Cartilage oligomeric matrix protein: a novel marker of cartilage turnover detectable in synovial fluid and blood. Br J Rheumatol 1992;31:583–91.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
30. Struglics A, Larsson S, Hansson M, Lohmander LS. Western blot quantification of aggrecan fragments in human synovial fluid indicates differences in fragment patterns between joint diseases. Osteoarthritis Cartilage 2009;17:497–506.
31. Struglics A, Hansson M. Calpain is involved in C-terminal truncation of human aggrecan. Biochem J 2010;430:531–8.
32. Lark MW, Gordy JT, Weidner JR, Ayala J, Kimura JH, Williams HR, *et al*. Cell-mediated catabolism of aggrecan. Evidence that cleavage at the “aggrecanase” site (Glu373-Ala374) is a primary event in proteolysis of the interglobular domain. J Biol Chem 1995;270:2550–6.
33. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta 1967;18:267–73.
34. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FP, *et al*. A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. Matrix Biol 1997;16:233–43.
35. Prophet EB, Mills, B., Arrington, JB, Sobin, LH., editor. Laboratory Methods in Histotechnology. Washington, 1992
36. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 1971;53:523–37.
37. Multanen J, Rauvala E, Lammentausta E, Ojala R, Kiviranta I, Hakkinen A, *et al*. Reproducibility of imaging human knee cartilage by delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) at 1.5 Tesla. Osteoarthritis Cartilage 2009;17:559–64.
38. Venn M, Maroudas A. Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. I. Chemical composition. Ann Rheum Dis 1977;36:121–9.
39. Maroudas A, Evans H, Almeida L. Cartilage of the hip joint. Topographical variation of glycosaminoglycan content in normal and fibrillated tissue. Ann Rheum Dis 1973;32:1–9.
40. Squires GR, Okouneff S, Ionescu M, Poole AR. The pathobiology of focal lesion development in aging human articular cartilage and molecular matrix changes characteristic of osteoarthritis. Arthritis Rheum 2003;48:1261–70.
41. Allen RG, Burstein D, Gray ML. Monitoring glycosaminoglycan replenishment in cartilage explants with gadolinium-enhanced magnetic resonance imaging. J Orthop Res 1999;17:430–6.
42. Williams A, Oppenheimer RA, Gray ML, Burstein D. Differential recovery of glycosaminoglycan after IL-1-induced degradation of bovine articular cartilage depends on degree of degradation. Arthritis Res Ther 2003;5:R97–R105.
43. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM. The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. Arthritis Rheum 2000;43:2202–10.
44. Tortorella MD, Liu RQ, Burn T, Newton RC, Arner E. Characterization of human aggrecanase 2 (ADAM-TS5): substrate specificity studies and comparison with aggrecanase 1 (ADAM-TS4). Matrix Biol 2002;21:499–511.
45. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 2003;5:94–103.
46. Ilic MZ, Handley CJ, Robinson HC, Mok MT. Mechanism of catabolism of aggrecan by articular cartilage. Arch Biochem Biophys 1992;294:115–22.
47. Tiderius C, Hori M, Williams A, Sharma L, Prasad PV, Finnell M, *et al*. dGEMRIC as a function of BMI. Osteoarthritis Cartilage 2006;14:1091–7.
48. Kulmala KA, Korhonen RK, Julkunen P, Jurvelin JS, Quinn TM, Kroger H, *et al*. Diffusion coefficients of articular cartilage for different CT and MRI contrast agents. Med Eng Phys 2010;32: 878–82.
49. Silvast TS, Jurvelin JS, Lammi MJ, Toyras J. pQCT study on diffusion and equilibrium distribution of iodinated anionic contrast agent in human articular cartilage – associations to matrix composition and integrity. Osteoarthritis Cartilage 2009;17:26–32.
50. Lammentausta E, Kiviranta P, Nissi MJ, Laasanen MS, Kiviranta I, Nieminen MT, *et al*. T2 relaxation time and delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) of human patellar cartilage at 1.5 T and 9.4 T: relationships with tissue mechanical properties. J Orthop Res 2006;24:366–74.