Human aggrecanase generated synovial fluid fragment levels are elevated directly after knee injuries due to proteolysis both in the inter globular and chondroitin sulfate domains

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SUMMARY

Objective: To examine different aggrecanase generated fragments in synovial fluid (SF) from patients with acute and chronic knee injuries and from knee healthy subjects.

Methods: We prepared SF-D1 samples from acute (n = 35) and chronic (n = 35) knee injury patients and knee healthy subjects (n = 10). Aggrecan fragments were analyzed in the SF-D1 samples by quantitative (G1, ARGs, KEEE and G3 antibodies) and non-quantitative (GRGT and AGEG antibodies) Western blot.

Results: ARGs-SELE, ARGs-chondroitin sulfate (CS)1, GRGT-, GLGS- and AGEG-G3 fragments were the main ARGs and G3 fragments in injured and reference samples. In the acute injury samples the concentrations of these fragments were increased compared to the reference, and the level of the ARGs-SELE remained elevated for at least 2 years after the joint injury. Both SF ARGs fragments and aggrecanase generated G3 fragments had high sensitivity and specificity as biomarkers in distinguishing injured from healthy knee joints, although the ARGs fragments had higher area under the receiver operating characteristic curve (AUC) values for injuries (74–86%) than the G3 fragments (AUC values 63–68%).

Conclusion: Our results suggest that during the acute phase after knee injury there is an increased aggrecanase activity against both the interglobular domain (IGD) and the CS2 cleavage sites of joint cartilage aggrecan. This increase in SF aggrecanolytic fragments is present for several years after the injury. SF ARGs fragments are better biomarkers than the aggrecanase generated G3-fragments in distinguishing injured from healthy knee joints.

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Introduction

Articular cartilage degradation is a hallmark of arthritis and joint injuries, involving an early loss of aggrecan fragments into synovial fluid (SF)1–3. Although both matrix metalloproteases (MMPs) and calpains have shown to cleave aggrecan4, the major proteases responsible for pathologic aggrecan degradation are the aggrecanases4–6. Aggrecanase involvement in human arthritis and knee injuries was first shown by the cleavage in the aggrecan interglobular domain (IGD) at the TEGE392 - 393 ARGS site5,6, while aggrecanase cleavage in the chondroitin sulfate (CS) 2 region at SELE1564 - 1565 GRGT, KEES1731 - 1734 GLGS, TAQE838 - 839 AGEG and ISQE1938 - 1939 LQQR sites was first shown in bovine explant cultures7,8 and later verified in bovine in vitro studies9,10. MMP proteolysis in the IGD at the IPEN360 - 361 FFGV site was first shown in pig11 and human12 aggrecan, while MMP cleavage in the CS1 region was first shown in bovine aggrecan13.

Several papers have reported on proteoglycan degradation taking place following knee joint injuries1–3,14,15, but there are only few papers providing details of the fragments generated during aggrecan turnover16–18.

Quantitative analysis of aggrecan proteolytic fragments has been conducted by enzyme-linked immunosorbent assay (ELISA)15,19–26, electrochemiluminescence27 and by mass spectrometry28. Western blot is considered a qualitative method, but is also readily used for quantitative measurements29–31. We recently developed a method for quantitative Western blot analysis of aggrecan fragments18,32. In analysis of biological events and in
searching for new biomarkers, Western blot has an advantage over conventional screening methods in differentiating fragments of different sizes sharing the same epitope, providing a detailed molecular pattern.

In the process of learning more about cartilage degradation following joint injuries, we here combine a detailed fragment analysis with the advantage of quantitative screening in Western blot to examine aggregan fragments in SFs from patients with acute and chronic knee joint injuries and from knee healthy subjects. The results provide a detailed mapping and quantitation of aggreganase generated fragments present in SF after human joint injuries.

Materials and methods

Antibodies and enzymes

Human recombinant ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs, aggreganase-1) and aggregan neo-specific anti-ARGSVILTKV monoclonal antibody (characterized — Supplementary data Fig. S1; Refs. 17,22) were kind gifts from M Pratta and Dr. S Kumar (GlaxoSmithKline). Polyclonal antibodies and enzymes generated fragments present in SF after human joint injuries. (Table I). Some of these SF samples have been used in previous biomarkers studies3. All procedures were approved by the ethics review committee of the Medical Faculty of Lund University.

Human SF samples

A SF osteoarthritis (OA) —pool was made as described33. SF was individually collected from 70 patients with knee injuries (35 acute and 35 chronic) and from 10 knee healthy reference (REF) subjects (Table I). Some of these SF samples have been used in previous biomarkers studies3. All procedures were approved by the ethics review committee of the Medical Faculty of Lund University.

Aggrecan SF-D1 isolation from SF

Mini SF-D1 preparations, collecting the lower half of the fractions, were done in the presence of protease inhibitors from 35 acute injury (Al), 35 chronic injury (Ci) and 10 REF subjects as described16. Sulfated glycosaminoglycan (sGAG; Alcian blue precipitation) and protein (Pierce Micro BCA™ Assay) analysis of SF and SF-D1 samples were conducted as described17. The average sGAG yield in the SF-D1 samples was 77% [standard deviation (SD) = 15, n = 79]. There was no correlation (r5 = 0.062, P = 0.59; n = 79) between the sGAG yield (range 45–124%) in SF-D1 and the amount SF sGAG loaded (16–546 µg) on the CsCl gradients, suggesting that the sGAG yields are not a result of differences of sGAG loaded. The SF-D1 samples were deglycosylated by chondroitinase ABC, keratanase and keratanase II and stored in lithium dodecyl sulfate (LDS)—sample buffer (Invitrogen) at −20°C17.

Quantification of aggrecan G1, ARGS, KEEE and G3 fragments by Western blot

ARGS standard was made as described18, by ADAMTS4 digestion of human OA cartilage A1D1 fraction, and the standard was thereafter deglycosylated17. The digest was assessed for complete conversion by Western blot using G1, ARGS and TEGE antibodies (data not shown). The human OA cartilage A1D1 fraction, which the ARGS standard was made from, contained no ARGS fragments in vivo33 and only 39% of full length G1–G3 monomers17. Therefore we used an aggregan molecular weight of 1.5 × 106 g/mol (i.e., for total protein sequence plus sGAG) which corresponds to 0.567 nmol ARGS fragments per mg aggregan dry weight.

A bovine cartilage aggrecan A1D1 fraction, prepared from calf (8–14 months old) metacarpophalangeal joints17, was used as G1 and G3 standards. This bovine A1D1 fraction contained 76.3% (mean value; SD) = 9%, n = 9 WB experiments) of full length monomers (G1–G3 fragments), and by using full length molecular weight of 2.5 × 106 g/mol (=0.4 nmol/mg), we assumed that the G1 and G3 standards contained 0.305 nmol (i.e., 0.763 × 0.4 nmol/mg) G1–G3 fragments per mg aggregan dry weight.

Since no KEEE standards were available, the SF D1 OA pool was used as a control sample for the quantification of KEEE fragments, expressing the data as relative units of the control sample. Subject SF D1 and the OA pool were run on same gels. Due to shortage of subject SF-D1 samples, only one Western blot experiment (with all available D1-samples) was run for detection of KEEE fragments.

Deglycosylated SF D1 samples and standards (i.e., ARGS, G1 and G3 standards; three different concentrations per gel) were denatured as described17, and together with Molecular weight markers (10–250 kDa; Precision Plus Protein Standards BIO RAD) separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 3–8% Tris-acetate midi-gels (26-wells, Invitrogen). The proteins were electrorephoretically transferred at 40 V for 70 min onto polyvinylidene fluoride (PVDF) membranes (BIO RAD #1620230) using a Criterion Blotter (BIO RAD #170–4070) and cooled 1 × NuPAGE transfer buffer (Invitrogen #NP0006) containing 10% methanol and 0.01% w/v SDS. Immuno-reactions were done as described17, using anti-G1 (1.5 µg/ml), anti-ARGS (5.3 µg/ml), anti-

<table>
<thead>
<tr>
<th>Study groups sub-groups</th>
<th>n</th>
<th>% male</th>
<th>Age in years, mean (range)</th>
<th>Time in weeks between injury and sampling, mean (range)</th>
<th>OA scores, range</th>
</tr>
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<td>REF</td>
<td>10</td>
<td>90</td>
<td>34 (19–58)</td>
<td>Na</td>
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<tr>
<td>Al 0–12 weeks</td>
<td>35</td>
<td>71</td>
<td>31 (20–61)</td>
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<td>1–5</td>
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<td>Al 0–2 weeks</td>
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<td>77</td>
<td>32 (20–61)</td>
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<td>1–4</td>
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<tr>
<td>Al 2–12 weeks</td>
<td>11</td>
<td>73</td>
<td>30 (20–60)</td>
<td>5.4 (2.4–11.9)</td>
<td>1–5</td>
</tr>
<tr>
<td>Cl 52 weeks</td>
<td>35</td>
<td>77</td>
<td>37 (19–59)</td>
<td>278.1 (52.1–1,941)</td>
<td>1–7</td>
</tr>
<tr>
<td>Cl 52–104 weeks</td>
<td>12</td>
<td>83</td>
<td>36 (19–52)</td>
<td>67.3 (32.7–103.4)</td>
<td>1–3</td>
</tr>
<tr>
<td>Cl &gt; 104 weeks</td>
<td>23</td>
<td>74</td>
<td>37 (21–59)</td>
<td>268.4 (104.4–1,941)</td>
<td>1–7</td>
</tr>
</tbody>
</table>

Study groups: Al (anterior cruciate ligament tear, meniscus tear, collateral ligament tear, osteochondral fracture, 0–12 weeks from injury to sampling); Cl (same injuries as Al, 52–1,941 weeks between injury and sampling); REF (no history of joint injury and no known joint pathology). OA score50. Na, not applicable.
KEEE (sera, 1:1,000) and anti-G3 (2 μg/ml) antibodies together with secondary peroxidase conjugated antibodies of goat anti-mouse IgG (20 ng/ml) and goat anti-rabbit IgG (13.3 ng/ml). The immunobands were visualized using ECL Plus Amersham Bioscience (ARGS and KEEE) or SuperSignal West Femto Pierce (G1 and G3) together with luminescence image analyzer (Fujifilm LAS-1000). The positioning and analysis of regions of interest of individual bands were done using Fujifilm software Image Gauge version 3.2.

The linearity of the ARGS, G1 and G3 standards (STD) was (sGAG per lane): 0.03–4 μg (R² = 0.99), 0.1–3 μg sGAG (R² = 0.99) and 0.05–1.5 μg (R² = 0.95), respectively. Due to lack of space on gels, only three concentrations of standards were run per gel (sGAG per lane): ARGS-STD = 0.1, 0.5 and 1.5 μg; G1-STD = 0.5, 1 and 2.2 μg and G3-STD = 0.2, 0.6 and 1 μg sGAG. SF-D1 samples which had signals outside the range of the signal of the standards were rerun on gels containing a broader signal range of the standards, so that all the SF-D1 samples had signals which were within the linearity of the corresponding standards.

Statistics

Kruskal–Wallis one way analysis of variance on ranks was used to avoid mass significance due to multiple group comparisons. If significances were found, then Mann–Whitney rank sum tests for analysis of unmatched pairs were run. For correlation analysis, Spearman rank order correlation (rs) was used. P-values < 0.05 were considered significant. Receiver operating characteristic (ROC) curve analysis was done with Statistical Package for Social Sciences (SPSS) software version 17.0.

Results

Quantitative Western blot screening of ARGS and G3 fragments

The aggrecan fragments detected by Western blot in the subjects’ SF-D1 fractions and discussed here are illustrated in Fig. 1. For each of the ARGS and G3 antibodies each subject’s SF-D1 sample, together with standards, were analyzed three times by SDS-PAGE and immuno blotting (i.e., n = 3 different Western blot experiments). For each subject; the mean, median, SD and coefficient of variation (CV) of aggrecan fragment concentrations were calculated. All data were corrected for the sGAG yields in the SF-D1 preparations, assuming that identical types of fragments were present in the recovered vs unrecovered material.

The within-patient CVs (based on the triplicates) for the total ARGS signal varied between the subjects, with an overall average (n = 77 subjects) of 25%. The average CV for ARGS regions A and B was 40% and 29%, respectively (Supplementary data Table SI). Similar analysis of the G3 aggrecan fragments showed average CVs between 41 and 64% (Supplementary data Table SI). Due to the high CV values, the median value for each patient was used for the calculation of group medians used in the comparisons between diagnostic groups.

SF aggrecanase generated ARGS fragments are increased following injury

Aggrecan fragments were purified by CsCl density centrifugation from SF of individuals, collecting the D1-fractions from the

Fig. 1. Aggrecan fragments detected by Western blot in the SF-D1 fractions. A schematic illustration of full length aggrecan: composed of three globular domains (G1, G2 and G3), an (between G1 and G2) IGD, a KS enriched region and two CS (CS1 and CS2) regions; and fragments of aggrecan detected by Western blot in the SF-D1 fractions of subjects. G1 fragments: region-A (>250 kDa), -B (150–250 kDa) and -C (100–150 kDa). ARGS fragments: region-A (280–310 kDa) and -B (120–160 kDa), and ARGS band-a1 (390 kDa). KEEE fragments: band-b (CS1-KEEE, 124 kDa) and -c (GRGT-KEEE, 50 kDa). G3 fragments with aggrecanase generated N-terminals: band-a (GRGT-G3, 214 kDa), -b (GLGS-G3, 171 kDa), -c (AGEG-G3, 137 kDa) and -d (LGQR-G3, 103 kDa). Aggrecanase cleavage sites are shown above.
lower half of the gradient. The Western blot showed that the D1 samples contained two ARGS fragment regions termed A (280–310 kDa) and B (120–160 kDa) (Supplementary data Fig. S2). The A-region contained 393ARGS-CS1/CS2 fragments—mainly the aggrecanase generated 393ARGS-SELE1564 fragment (310 kDa), but some of the injury SF-D1 samples also contained a 280393ARGS-CS1 fragment with a C-terminal previously identified as calpain generated EDLS1472 (Refs.18,32). The B-region contained 393ARGS-CS1 fragments with multiple C-terminals previously estimated as MMP generated GV(G/E)D9521409 (Refs.17,18). In addition, approximately 50% of the injury patients (acute and chronic) and 10% of the knee healthy controls (reference) had an additional 390 kDa ARGS-CS2 fragment (band-a1) with an estimated (using a calculation model)14 C-terminal of TAQE generated by an aggrecanase cut at the TAQE1838Y1839AGEG site (Supplementary data Fig. S2).

In the AI groups the total ARGS concentration was 33 times (AI, 0–2 weeks) and five times (AI, 2–12 weeks) higher than in the REF group, while in the CI groups (52–104 weeks and >104 weeks) the total ARGS signal did not differ from the reference level [Fig. 2(a), Table II]. In the injury groups, ARGS fragments constituted 5–28% of total aggrecan, and were significantly increased for many years compared to reference levels (Table III). Both ARGS region-A and -B concentrations were increased in the AI groups (99 times for ARGS region-A in the 0–2 weeks group). While the ARGS region-A level

![Graph](image_url)

**Fig. 2. Concentration of aggrecan ARGS-fragments in the study groups.** Total ARGS, ARGS region-A (280–310 kDa) and region-B (120–160 kDa) were quantified in SF-D1 samples by Western blot using ADAMTS4 digested human cartilage-A1D1 fraction as ARGS-standards. The boxes define the twenty-fifth and seventy-fifth percentile with a line at the median, error bars define the tenth and ninetieth percentile and circles represent individual outliers. * Indicates significance (P < 0.05) between injury and reference groups. Actual P-values are shown in Table II. Diagnostic groups are as in Table I.

<table>
<thead>
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<th>Diagnostic groups</th>
<th>n</th>
<th>ARGS region A/B</th>
<th>P-values</th>
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<td>REF</td>
<td>10</td>
<td>280–310 kDa</td>
<td>0.30 (0.1–0.74)</td>
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<tr>
<td>AI 0–12 weeks</td>
<td>35</td>
<td>640 (0–2.13)</td>
<td>0.64 (0–2.13)</td>
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<tr>
<td>CI &gt; 52 weeks</td>
<td>32</td>
<td>620 (0.1–3.53)</td>
<td>0.62 (0.1–3.53)</td>
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</table>

### Table II

Aggrecan ARGS fragments in human SF samples. Concentration (in pmol/ml SF) of total ARGS, ARGS region-A and -B was measured in SF-D1 samples using Western blot, ARGS antibody and human ARGS standards. Concentrations, expressed as median (min–max range) values in diagnostic groups. Median values of ARGS were normalized (Norm) against the reference group. P-values, significant analysis (Mann–Whitney) of injury vs reference group. Diagnostic groups were according to Table I.

<table>
<thead>
<tr>
<th>Diagnostic groups</th>
<th>n</th>
<th>TotalARGS Norm</th>
<th>P-values</th>
<th>ARGS region-A Norm</th>
<th>P-values</th>
<th>ARGS region-B Norm</th>
<th>P-values</th>
</tr>
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<tbody>
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<td>REF</td>
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<td>1.3 (0.5–12.0)</td>
<td>0.15 (0.1–1.6)</td>
<td>0.75 (0.2–9.5)</td>
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<td></td>
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<tr>
<td>AI 0–2 weeks</td>
<td>24</td>
<td>44.7 (7.6–193.0)</td>
<td>14.6 (0.4–106.3)</td>
<td>23.0 (3.0–56.9)</td>
<td>0.001</td>
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<tr>
<td>AI 2–12 weeks</td>
<td>11</td>
<td>6.6 (2.1–53.4)</td>
<td>2.1 (0.2–20.2)</td>
<td>3.4 (0.5–29.7)</td>
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<tr>
<td>CI 52–104 weeks</td>
<td>12</td>
<td>2.7 (0.8–18.6)</td>
<td>0.63 (0.2–5.1)</td>
<td>0.78 (0.3–7.3)</td>
<td>0.004</td>
<td></td>
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</tr>
<tr>
<td>CI &gt; 104 weeks</td>
<td>20</td>
<td>1.3 (0.2–239.0)</td>
<td>0.24 (0.8–41.4)</td>
<td>0.59 (0.1–98.5)</td>
<td>0.004</td>
<td></td>
<td></td>
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### Table II

Aggrecan ARGS fragments in human SF samples. Concentration (in pmol/ml SF) of total ARGS, ARGS region-A and -B was measured in SF-D1 samples using Western blot, ARGS antibody and human ARGS standards. Concentrations, expressed as median (min–max range) values in diagnostic groups. Median values of ARGS were normalized (Norm) against the reference group. P-values, significant analysis (Mann–Whitney) of injury vs reference group. Diagnostic groups were according to Table I.

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<td>0.004</td>
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was increased up to 2 years after injury, the ARS group-B concentration had returned to the reference level in the chronic groups [Fig. 2(b and c), Table II]. The ARS region A/B ratio was higher in both AI and CI groups when compared to the reference [Fig. 2(d), Table II], and the level of the 390 kDa ARS-CS2 fragment (band-a1) was increased in the CI group compared to the reference (P = 0.007, data not shown).

These results confirm previous observations of a marked release of aggrecanase-generated ARS fragments in the acute phase following injury, and that ARS fragments represent a large proportion of total aggrecan in these SF samples. Further, although the overall SF ARS concentration did not significantly differ from reference level 2 years after injury, the differences in ARS region fragment A and B proportions between injury and control groups suggest a persistent change in aggrecan turnover many years after the joint injury.

**SF aggrecanase generated CS2-G3 fragments are increased following injury**

The G3 Western blot showed that the SF D1-fractions prepared from the subjects contained G1–G3 monomers (>400 kDa), and aggrecanase generated 214 kDa [GRGT-G3], 171 kDa CS2-G3, 137 kDa [AGEG-G3] and 103 kDa CS2-G3 fragments (Supplementary data Fig. S3). The GRGT and AGEG N-terminals of these CS2-G3 fragments were verified by neo-epitope antibodies, while the 103 and 171 kDa CS2-G3 fragments were estimated by calculation as [LGQR-G3] and [GLGS-G3], respectively (Fig. 3).

The SF concentrations of full length G1–G3 monomers did not differ significantly between the injury and reference groups [Fig. 4(a), Table IV]. The LGQR-G3 fragment was only found in some of the injured and none of the uninjured samples, and was therefore not analyzed further (data not shown). On the other hand the GRGT-, GLGS- and AGEG-G3 were the dominant G3 fragments in both injured (mean = 79% of total G3-signal, SD = 21%, n = 68) and reference (mean = 70% of total G3-signal, SD = 26%, n = 9) samples, but the SF concentrations of the aggrecanase generated GRGT-, GLGS- and AGEG-G3 fragments were increased in the AI samples compared to the reference, where the AGEG-G3 fragment increased most (12 times, in the 0–2 weeks AI group) [Fig. 4(b–d), Table IV]. At 2 years after injury the SF concentrations of these G3-fragments did not differ from the reference levels ([Fig. 4(b–d), Table IV].

The relative amounts of aggrecanase generated G3 fragments in the SF reference fluids were [GLGS-G3] > [GRGT-G3] > [AGEG-G3], although in the 0–2 weeks AI group the AGEG-G3 was the dominant G3 fragment (Table IV). The relative proportion of these G3 fragments differed between the AI group (0–12 weeks) and the reference (lower proportions of the GRGS- and GLGS-G3 fragments and higher proportion of AGEG-G3), but the proportions were normalized in the CI group (>52 weeks) [Fig. 4(e)].

These results suggest that aggrecanase activity against sites in the CS2 region is increased directly after injury, which results in high SF concentrations of GRGT-, GLGS- and AGEG-G3 fragments. Further, the SF concentrations of these G3-fragments are reduced to reference levels at 2 years from injury.

**ARGS and G3 fragments as biomarkers for joint injuries**

There were no differences in the SF median total ARS concentration (measure of the aggrecanase cut in the IGD) between men and women comparing all (P = 0.576) or only injured subjects (P = 0.383). Similarly, there were no differences between genders in the SF median GRGT-, GLGS- and AGEG-G3 fragment concentrations (measure of aggrecanase cuts in the CS2 region) of all (P = 0.138, = 0.155 and = 0.208 respectively) or only injured subjects (P = 0.102, = 0.115 and = 0.150 respectively).

The sensitivity and specificity was high for aggrecanase generated SF ARS (ARGS region-A, -B and total ARS) and G3 (GRGT-G3, GLGS-G3 and AGEG-G3) fragments as markers for joint injuries (i.e., AI alone or AI + CI) (Table V). There were no major differences
between the ARGS markers (ARGS region-A, -B and total ARGS), although all of them had higher area under the ROC curve (AUC) values for AI (94\(\pm\)98\%) and for AIs plus CIs (74\(\pm\)86\%) compared to corresponding AUC values (AI, 72\(\pm\)83\%; AI + CI, 63\(\pm\)68\%) of the G3 fragments (Table V).

These results suggest that of the high Mw aggrecan fragments found in SF-D1 samples, ARGS fragments are better biomarkers in distinguishing injured from healthy knee joints compared to aggrecanase generated G3 fragments (i.e., GRGT-G3, GLGS-G3 and AGEG-G3), and that gender has no influence in these comparisons.

Differences in the high Mw SF aggrecan fragment pattern between injured and knee healthy subjects visualized by Western blot

The 80 SF-D1 samples were screened for high Mw (i.e., sGAG containing) aggrecan fragments by Western blot with antibodies recognizing G1 and G3 domains, and with antibodies against aggrecanase generated neoepitope ARGS and KEEE.

As shown above the aggrecanase generated ARGS region-A and -B, and band-a1, were all increased compared to the reference following injury [Fig. 5(a)]. Similarly, the GRGT-G3, GLGS-G3 and

Fig. 4. Concentration of aggrecan G3-fragments in the study groups. G3 fragments were quantified in SF-D1 samples by Western blot using bovine cartilage-AID1 fraction as G3-standards. SF concentrations of G1–G3 (a), GRGT-G3 (b), GLGS-G3 (c), AGEG-G3 (d) are shown. The proportion of G3 fragments was calculated (from mol concentrations) as the ratio of a G3 fragment over the sum of GRGT-G3 (band-a), GLGS-G3 (band-b) and AGEG-G3 (band-c) fragments (e). The boxes define the twenty-fifth and seventy-fifth percentile with a line at the median, error bars define the tenth and ninetieth percentile and circles represent individual outliers. *Indicates significance (P < 0.05) between injury and reference groups. Actual P-values for (a) – (d) are shown in Table IV. Diagnostic groups are as in Table I.
AGEG-G3 fragments were increased following injury [Fig. 5(a)]. A 124 kDa CS1–KEEE fragment (band-b) with an estimated MMP generated N-terminal of ISGL, and a 50 kDa aggrecanase generated GRGT-KEEE fragment (band-c) were also significantly increased during injury [Fig. 5(a), Supplementary data Fig. S4(a)]. G1 fragments in region-A (Mw > 250 kDa); G1–G3 monomers and G1–CS2 with aggrecanase generated KEE/SELE and region-B (Mw = 150–250 kDa); G1-CS1 with an estimated MMP generated C-terminal of GVED were the dominant G1-fragments in the reference group (data not shown). The concentrations of G1-region- B fragments were significantly reduced in the injury groups, while G1 region-C fragments (Mw = 100–150 kDa); G1-keratan sulfate (KS) with unknown C-terminal (K) were significantly increased during Al, all compared to the reference group [Fig. 5(a), Supplementary data Fig. S4(b)].

These results suggest that there is a high Mw (>100 kDa) aggrecanase generated SF aggrecan injury pattern which is significantly different from the turnover pattern of the REF group [illustrated in Fig. 5(b)]. This difference in pattern is due to elevated aggrecanase proteolysis against both the IGD and CS2 sites of aggrecan in injured joints.

Discussion
We have previously presented a quantitative Western blot method for aggrecan fragments where we analyzed a few SF-D1 samples from five diagnostic groups [18]. In the present paper we have considerably extended the patient numbers, focusing on AlS and CIs in comparison with knee healthy subjects, and in a detailed analysis provide quantitative and qualitative information on additional aggrecan fragments.

CS1 purification of SF aggrecan together with Western blot analysis allowed quantitative detection of high Mw (>100 kDa) aggrecanase generated fragments from seventy knee injured and ten knee healthy subjects. These measurements showed that directly after injury (within 2 weeks) there was an increased aggrecanase cleavage in the aggrecan IGD, generating ARGS-SELE and ARGS-CS1 fragments. Similarly increased aggrecanase cuts were seen against the aggrecan CS2 sites generating GRGT-CS3, GLGS-G3 and AGEG-G3 fragments. The levels of the aggrecan generated G3 fragments were normalized 2 years after injury, although there was still a difference in the ARGS-SELE/ARGS-CS1 ratio between injury and reference. Further, the SF patterns of G1 and KEEF fragments were also markedly changed after injury. Together these data show that there is a change in the aggrecan turnover lasting many years after knee injury.

Several of the aggrecan fragments found in these SF-D1 samples were generated by aggrecanase digestion in combination with cuts by MMPs (e.g., ARGS-GVED, ISGL-KEEE) or calpains (ARGS-EDLS). Also, MMP generated FFGV fragments have been detected in SF-D1 samples [17], but calpain generated PGVA and GDLS fragments [32] were not found in these SF-D1 samples (data not shown). Even though some of the MMP and calpain cuts of aggrecan might be involved in normal turnover, it is also possible that these enzymes have an active role in the degradation processes following joint injuries.

Knee injuries are accompanied by increased SF levels of pro-inflammatory cytokines [22–36] and increased levels of ARGS fragments have been observed in explant studies when stimulating cartilage with cytokines [22,36–38]. Mechanically injured bovine cartilage discs have an increased mRNA expression of cartilage matrix degrading enzymes [30] which result in proteoglycan and collagen degradation in these tissues [40,41]. Increased SF levels of proteoglycans and/or aggrecan [3] and the aggrecan neoeptope ARGS15,18 directly after knee injury (i.e., acute phase 0–12 weeks) are well established. Our results (Figs. 2 and 3, Table II) support these findings, which are now extended by our Western blot data showing that concentrations of both ARGS-SELE and ARGS-CS1 fragments are increased, and that the ratio of these fragments is constantly higher in the injury group compared to reference for many years after injury.

SF-ARGS ELISA measurements can distinguish diseased joints (i.e., arthritis and joint injury) from reference joints with high sensitivity and specificity [15], and a similar differentiation in

### Table IV

Aggrecan G3 fragments in human SF samples. Concentration (in pmol/ml SF) of G1–G3, GRGT-G3, GLGS-G3 and AGEG-G3 fragments was measured in SF-D1 samples using Western blot, G3 antibody and bovine G3 standards. Concentrations, expressed as median (min–max range) values in diagnostic groups. Median values of G3 fragments were normalized (Norm) against the reference group. P-values, significant analysis (Mann–Whitney) of injury vs reference group. For the GRGT-G3 fragment; the Kruskal–Wallis test of random sampling variability was not significant (the P-values were therefore not determined, Nd), although the Kruskal–Wallis test was significant for time periods Al (0–12 weeks) and CI (>52 weeks) where the Al vs reference differed significantly (P = 0.048). Diagnostic groups were according to Table I

<table>
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<tr>
<th>Diagnostic groups</th>
<th>n</th>
<th>G1–G3</th>
<th>Norm</th>
<th>P-values</th>
<th>G1–G3</th>
<th>Norm</th>
<th>P-values</th>
<th>G1–G3</th>
<th>Norm</th>
<th>P-values</th>
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<tr>
<td>REF</td>
<td>9</td>
<td>1.4 (0.3–3.3)</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Al 0–2 weeks</td>
<td>24</td>
<td>0.36 (0.4–4.4)</td>
<td>0.3</td>
<td>0.129</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Al 2–12 weeks</td>
<td>11</td>
<td>1.2 (0.4–8)</td>
<td>0</td>
<td>0.939</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CI 52–104 weeks</td>
<td>12</td>
<td>1.9 (0.3–9.7)</td>
<td>1.4</td>
<td>0.337</td>
<td></td>
<td></td>
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<tr>
<td>CI &gt; 104 weeks</td>
<td>21</td>
<td>1.1 (0.8–5)</td>
<td>0.8</td>
<td>0.928</td>
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### Table V

Sensitivity and specificity of aggrecan generated SF ARGS and G3 fragments in distinguishing between injured and healthy knee cartilage. Concentrations (in pmol/ml SF) of ARGS region-A, ARGS region-B, total ARGS, GRGT-G3, GLGS-G3 and AGEG-G3 fragments were measured in SF-D1 samples using Western blot and human ARGS or bovine G3 standards. The data (in %): comparison between Al (0–12 weeks) and reference (healthy), and between Al (0–12 weeks) plus CI (>52 weeks) and reference. Diagnostic groups were according to Table I

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>AlS</th>
<th>AIs and CIs</th>
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<tr>
<td></td>
<td>AUC</td>
<td>Sensitivity</td>
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<tr>
<td>ARGS region A</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>ARGS region B</td>
<td>94</td>
<td>94</td>
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<tr>
<td>ARGS total</td>
<td>96</td>
<td>94</td>
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<tr>
<td>GRGT-G3</td>
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<td>71</td>
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<tr>
<td>GLGS-G3</td>
<td>75</td>
<td>83</td>
</tr>
<tr>
<td>AGEG-G3</td>
<td>83</td>
<td>69</td>
</tr>
</tbody>
</table>

Sensitivity – the proportion of positives (diseased) correctly identified by the test. Specificity – the proportion of negatives (healthy) correctly identified by the test.
total SF-ARGS was obtained by Western blot measurements when we compared injury group with a reference group (Table V). SF concentrations of the ARGS-SELE fragment showed the greatest difference between injured and control subjects, although this fragment was not a better biomarker than total ARGS in distinguishing injured from healthy subjects. This suggests that ARGS-ELISA26 and electrochemiluminescence methods27 measuring total ARGS are still the best methods for screening large numbers of samples.

The aggrecanase generated aggrecan fragment SF-pattern from injured knees (AI – CI) observed here resembles qualitatively the pattern observed in OA and acute arthritis (Supplementary data Figs. S2 and S3;16,17,33,34). Although the difference in aggrecanase generated fragment pattern between injury and REF was marked, no clear difference was seen between patterns observed early after injury compared to those later after injury.

The aggrecan fragments appearing in SF are proteolytic products of larger G1 fragments (e.g., G1—CS1, G1-SELE/KEEE and G1—G3) present in the cartilage extracellular matrix16,17,33,34. Many knee injuries are isolated meniscus tears or combinations of meniscus and anterior/posterior crucial ligament injuries43,44, and since the meniscus and the ligaments contain aggrecan21,45 it is plausible that these tissues may contribute to the overall high aggrecan fragment concentrations in SF following injury.

Proteolytic cleavage in the IGD of cartilage aggrecan releases the sGAG-containing C-terminal part of the molecule which mainly diffuses out into SF15,17,42 or into media in explant systems46,47. On the other hand, the N-terminal G1 fragments remain in the tissue or

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**Fig. 5. The pattern of high Mw SF aggrecan fragments from knee injury patients and reference subjects.** The high Mw (i.e., sGAG containing) aggrecan fragment pattern in SF-D1 fractions was analyzed, by Western blot, from knee injured patients (AI 0–12 weeks and CI > 52 weeks) and REF. (a) Characteristic high Mw G1, ARGS, KEEE and G3 aggrecan fragment pattern from specific subjects are presented. (b) Illustration of relative levels of high Mw aggrecan fragments between knee healthy controls and knee injured patients found in SF-D1 samples by Western blot. A significant increase in one group is illustrated in black, while the rest of the fragments is shown in gray. G1 strips (3 µg sGAG per lane): region-A (G1—G3 monomer and G1-CS2, 250 kDa), region-B (G1—CS3, 150–250 kDa) and region-C (G1—KS, 100–150 kDa). ARGS strips (0.8 µg sGAG per lane): region-A (ARGS-CS1/CS2, 280–310 kDa), region-B (ARGS-CS1, 120–160 kDa) and band-a (ARGS-CS2, 390 kDa). KEEE strips (2 µg sGAG per lane): region-A (G1-KEEE and IGD-KEEE, 340–370 kDa), band-b (CS1-KEEE, 124 kDa) and band-c (GRGT-KEEE, 50 kDa). G3 strips (1.5 µg sGAG per lane): band-1 (G1—G3 monomer, > 400 kDa), band-a (GRGT-G3, 214 kDa), band-b (GLGS-G3, 171 kDa) and band-c (AGEG-G3, 137 kDa). The Western blot for these aggrecan fragments have been described previously33.
are internalized by chondrocytes\textsuperscript{46,48,49}. The injurious joint insult induces an increase in pro-inflammatory cytokine levels in the damaged joint tissues, which in turn up-regulates aggrecanolytic enzymes, resulting in increased aggrecan degradation. Although aggrecanase activity within the CS2 region has been proposed to be a result of aggrecan maturation\textsuperscript{6,17,32}, it is interesting to note that these cuts in CS2 were also increased as a result of injury. An explanation for why there is an increased cut in the CS2 region after injury could be that aggrecanase digestion in CS2 is more preferred than cuts in the IGD as shown for bovine\textsuperscript{19} and human aggrecan (our data — not shown), and/or that cuts in CS2 facilitate the removal of high Mw aggrecan fragments from injured tissues.

This study has some general limitations: (1) There is a big difference in sGAG yield in these preparations which could be explained by heterogeneous SF samples with differences in protein and sGAG concentrations and in viscosities. (2) Enrichment of aggrecan fragments is necessary before analysis by SDS-PAGE and Western blot. Here we used dissociative CsCl centrifugation assuming that the proportion of high Mw (>100 kDa) aggrecan fragments present in the D1 fraction reflects the high Mw in vivo content of SF. (3) There might be differences in the efficiency of electro-transfer of aggrecan fragments with different size and charge, and any differences in the affinity of the antibodies for fragments of different size carrying the same epitope would affect the detected ratio of the signals. (4) The Western blot quantification method has a higher CV compared to methods such as ELISA. Therefore the comparisons between groups were done using subject median values instead of means. (5) We have used human ARGS- and bovine G3-standards (purified from cartilage) in the quantification studies, which make a comparison between ARGS and G3 fragment concentrations difficult. This could be overcome if instead using human recombinant aggrecan fragments as standards.

Our study also has some strengths. The advantage of the Western blot quantification method over more regular methods such as ELISA is that it discriminates between different proteolytic fragments carrying the same epitope, and therefore allows a more detailed quantitative analysis of the products of aggrecan proteolysis.

We have shown that in patients suffering from acute knee joint injuries, the SF concentrations of several different aggrecanase generated fragments are markedly increased. Both aggrecanase cuts in the IGD and cuts in the aggrecanase CS2 sites are increased. This change in fragment patterns is present several years after the injury. The continued change in aggrecan turnover pattern may contribute to the frequent development of OA following joint injuries\textsuperscript{43}.

Author contributions

All authors have substantially contributed to the conception and design of the study, acquisition of data, or analysis and interpretation of data. All authors have participated in the writing process and approved the final version of the manuscript. André Struglics (andre.struglics@med.lu.se) takes responsibility for the integrity of the work.

Role of the funding sources

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Conflict of interest

The authors report no conflict of interest with this study.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.joca.2011.05.006.

References


