Juvenile idiopathic arthritis patients have a distinct cartilage and bone biomarker profile that differs from healthy and knee-injured children

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Abstract

Objective

Joint destruction is a hallmark of juvenile idiopathic arthritis (JIA). Clinical evaluation and radiographic imaging are current methods to identify destruction. Biomarkers could aid an earlier and more sensitive diagnosis. Our aim was to investigate levels of bone and cartilage degradation biomarkers in JIA patients, compared to healthy children or juveniles with knee injuries.

Methods

Triple-paired synovial fluid, plasma and urine samples from 29 JIA patients were compared to 61 plasma samples from healthy children and synovial fluid from 41 knee-injured juveniles. Cartilage biomarkers ARGS neoepitope of aggrecan (ARGS), cartilage oligomeric matrix protein (COMP), type II collagen epitope (C2C), bone biomarkers N-terminal type I collagen cross-linked telopeptide (NTX-I) and tartrate-resistant acid phosphatase 5b (TRAP5b) were analysed by immunoassays.

Results

Plasma levels of ARGS, C2C, COMP and TRAP5b were increased in JIA compared to healthy children. Compared to knee-injured juveniles, synovial fluid C2C and TRAP5b were increased in JIA, while ARGS and COMP were decreased. For JIA patients, local (synovial fluid) and systemic (plasma/urine) levels of bone biomarkers correlated positively; age correlated negatively to plasma levels of C2C and TRAP5b; no correlation was found between biomarkers and gender, affected joint count, disease duration or medication.

Conclusion

Elevated levels of destruction biomarkers in JIA compared to healthy children indicate a potential to serve as clinical tools for destructive joint disease. High levels of TRAP5b, NTX-I and collagen II in JIA in contrast to more pronounced aggrecan and COMP degradation in juvenile knee injuries, suggests that JIA patients have a unique biomarker pattern, different from healthy and knee-injured children.

Key words

juvenile idiopathic arthritis, biomarkers, destruction, bone, cartilage

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Introduction

Juvenile idiopathic arthritis (JIA), the most common chronic rheumatic disease of childhood, is characterised by prolonged synovial inflammation that may lead to permanent damage of articular cartilage and bone (1). JIA is divided into seven subtypes based on clinical features: oligoarthritis, rheumatoid factor (RF) positive polyarthritis, RF negative polyarthritis, psoriatic arthritis (PsA), enthesitis-related arthritis (ERA), systemic onset JIA (soJIA) and undifferentiated arthritis. Oligoarthritis and RF-negative polyarthritis account for the largest proportion of patients (2, 3). Besides cartilage and bone degradation, joint damage seen in JIA includes ankylosis, growth disturbances and joint malalignment. All joints can be affected but most common are ankle, wrist and hip joints together with temporomandibular joints. The advent of biological treatments has improved prognosis of JIA patients significantly, nevertheless, this patient group is still at risk of cartilage and bone erosion, which can lead to irreparable joint destruction or joint replacements in childhood (4, 5). Pro-inflammatory cytokines, in particular tumour necrosis factor (TNF) and Interleukin 1 (IL-1) are important mediators of disease progression. Regulatory cytokines and the presence of catabolic factors in the inflamed synovium may also have a major impact on destruction. Inflammation can be monitored by ultrasound, but only monitoring inflammatory parameters as a measure of disease activity is not sufficient in JIA (6). It is equally important to detect joint destruction as early as possible. Current methods to monitor destructive events are clinical evaluation in combination with radiographic imaging that identifies changes in the joint structure and joint space caused by loss of articular cartilage and bone. However, these methods are insensitive and mainly detect late stages of destruction (7, 8). Joint destruction often progresses from the cartilage surface to the subchondral bone and leads to structural degradation and release of molecular fragments of the joint into biologic fluids, which could be used as biomarkers (9). To date there are no validated biomarkers available for detecting initial stages of joint destruction in JIA before it has become radiologically visible. Such biomarkers would be helpful in monitoring therapy response, progression of joint damage and potentially predicting disease outcome.

Previously the biomarker research in JIA focused on different antibodies: RF, anti-nuclear antibodies and anti-citrullinated protein antibodies (ACPA) and on inflammatory markers such as erythrocyte sedimentation rate, C-reactive protein, cytokines and alarmins (10-16). The prognostic value of these are not comprehensive, there are indications that inflammatory markers may predict erosion (11) and that ACPAs may help to sub-diagnose JIA patients with bone involvement and erosions although with low sensitivity (15). Few investigations have been performed in order to define biomarkers of cartilage and bone degradation in JIA patients (17-22). In contrast, there are many studies investigating cartilage and bone biomarkers of joint destruction in other joint diseases in adults, including osteoarthritis (OA) and rheumatoid arthritis (RA) (23-29).

The present study is exploratory and hypothesis generating. Our primary aim was to investigate levels of several well-studied biomarkers for bone and cartilage degradation in a JIA cohort in comparison to healthy children or children with acute knee injuries. Our second aim was to evaluate suitable biofluids for detecting degradation biomarkers in JIA and finally to explore the potential influence of confounders such as age, gender, disease onset, disease duration, number of affected joints and medication at time of sampling. Chosen biomarkers are well-studied in other degenerating joint diseases, in particular OA (30). Degradation of cartilage was monitored by measuring ARGS neoepitope of aggrecan (ARGS-aggrecan), C2C neoepitope of collagen type II and cartilage oligomeric matrix protein (COMP). To monitor degradation of bone, N-terminal type I collagen cross-linked telopeptide (NTX-I) and tartrate-resistant acid phosphatase 5b (TRAP5b) were measured.

Materials and methods

Subjects and sampling

Triple-paired samples of synovial fluid, plasma and urine (sampling at the same time-point) were collected from 29 JIA patients with active disease at Astrid Lindgren's Children Hospital in Stockholm, Sweden as part of our JIA population based biobank JABBA (Juvenile Arthritis BioBank Astrid Lindgren's hospital). Mean patient age was 12.1 years, 62% were females and median disease duration was 2.3 years (Table I). Patients were enrolled and diagnosed according to International League of Associations for Rheumatology (ILAR) criteria, and JIA subtype distributions were as follows: oligoarthritis (n=20; 69%), polyarthritis (n=7; 24%), and enthesitis related arthritis (n=2; 7%) (Table I). Synovial fluid was collected in citrate tubes, plasma in EDTA tubes, and urine in tubes without any additives. Synovial fluid samples were centrifuged at 3000xg for 10 minutes to obtain cell-free samples. Urine samples were centrifuged at room temperature at 3000xg for 15 minutes. Samples were stored at -80ºC until analysis. From the Swedish Pediatric Rheumatology Quality Registry or medical records, disease-related patient data, including number of active joints, current and past medication, disease duration and disease onset was extracted. Plasma samples from 183 healthy reference subjects, without inflammatory or autoimmune diseases (4, 8 or 12 years old) were from a population-based cohort (Barnens hälsoundersökning) collected in the Stockholm region, *i.e.* the same region as the uptake of the JIA patients. Due to low sample volume, the healthy references were divided into three age and gender matched sub-cohorts (n=61) that had a mean age of 9.7 years and 58% were females (Table I).

From a knee injury cohort, 41 juvenile subjects were selected: mean age of 17 years (range 13.2–19.6) and 29% were female (Table I). From each of these subjects, synovial fluid had been aspirated on one occasion at various times after an acute knee injury (0–20 days), centrifuged at 3000g for 10 minutes at room temperature and then stored at -80ºC. All knee-injured subjects had haemarthrosis with the following magnetic resonance imaging (MRI) based diagnosis: anterior cruciate ligament rupture (n=21), posterior cruciate ligament rupture (n=1), meniscus injury (n=1), patella luxation (n=7), normal MRI (n=11).

Table I. Characteristics and demographics of the subject groups.

Study groups	Sex, % female	n.	Mean age in years at sampling (SD)	Disease duration, range (median)	Affected joints, n.	Samples, n. (biofluids)	Diagnosis	Medication
Juvenile injury	29	41	17.0 (1.8)	0 - 20 days (2.0)	1	41 (SF)	Knee injuries	Analgesics
Reference (all)	58	193	9.7 (3.3)	Na	Na	193 (P)	Healthy references	Na
Ref subgroups	*53–57*	*61*	*9.4–9.6 (3.3–3.4)*	*Na*	*Na*	*61 (P)*	*Healthy references*	*Na*
JIA	62	29	12.1 (3.5)	0–16.6 years (2.3)	1–10	23 (SF), 29 (P), 25 (U)	JIA	Mixed
Individual JIA subjects	Sex		Age	Disease years	Affected joints, n	Biofluids		
1	F	-	10.0	0.0	3	SF, P, U	Polyarthritis RF-	*None*
2	F	-	17.5	0.8	3	SF, P, U	Oligoarthritis	*None*
3	M	-	13.8	5.7	1	SF, P, U	Oligoarthritis	*None*
4	M	-	16.5	0.5	1	SF, P, U	Enthesitis related arthritis	*None*
5	M	-	16.6	3.3	1	SF, P, U	Oligoarthritis	Methotrexate
6	F	-	11.1	13.0	2	---, P,---	Polyarthritis RF-	Methotrexate
7	F	-	11.4	0.7	2	SF, P, U	Oligoarthritis	NSAID
8	F	-	11.4	9.7	1	SF, P, U	Oligoarthritis	Cortisone injection
9	F	-	12.0	13.0	4	---, P,---	Polyarthritis RF-	Humira
10	F	-	7.3	5.3	4	---, P, U	Polyarthritis RF-	Orencia
11	F	-	10.3	7.6	8	---, P,---	Oligoarthritis	Methotrexate
12	M	-	5.9	0.3	3	SF, P, U	Polyarthritis RF-	*None*
13	F	-	17.6	2.3	2	SF, P, U	Oligoarthritis	*None*
14	F	-	10.7	7.2	1	SF, P, U	Oligoarthritis	*None*
15	M	-	11.4	0.2	2	SF, P, U	Oligoarthritis	NSAID
16	M	-	14.8	0.6	3	SF, P, U	Oligoarthritis	*None*
17	F	-	12.6	8.9	2	SF, P, U	Polyarthritis RF-	Methotrexate
18	F	-	7.2	0.3	1	SF, P, U	Oligoarthritis	NSAID
19	F	-	13.4	0.2	1	SF, P, U	Oligoarthritis	*None*
20	F	-	12.0	10.3	8	---, P, U	Polyarthritis RF-	*None*
21	M	-	14.3	0.1	3	SF, P, U	Oligoarthritis	*None*
22	F	-	12.8	10.4	3	SF, P, U	Oligoarthritis	Methotrexate
23	M	-	6.2	2.3	10	SF, P, U	Oligoarthritis	Methotrexate
24	M	-	13.3	0.7	1	SF, P, U	Oligoarthritis	*None*
25	M	-	11.8	5.0	1	SF, P, U	Oligoarthritis	*None*
26	F	-	13.4	9.4	2	---, P,---	Oligoarthritis	Methotrexate
27	M	-	15.3	1.1	1	SF, P, U	Enthesitis related arthritis	Methotrexate
28	F	-	16.6	16.6	1	SF, P, U	Oligoarthritis	Cortisone injection
29	F	-	4.9	0.0	1	SF, P, U	Oligoarthritis	*None*

There was no difference in sex ratio between the healthy reference (n=61) and the JIA (n=29) groups (p between 0.391 and 0.672), nor between the JIA (n=23) and juvenile injury (n=41) groups (p=0.069). The subjects in the healthy reference groups (n=61) were younger (p=0.001) than the JIA group (n=29), while the juvenile injury group were older (p<0.001) than the JIA group (n=23). There was no difference in age (p between 0.504 and 0.850) or sex (p between 0.286 and 0.900) between the healthy reference group (n=193) and the sub-groups of healthy references (n=61). Of the JIA patients, 51.7% (15 of 29) were under medication, as specified in the medication column. Affected joints refer either to number of joints with arthritis according to ILAR criteria (for JIA) or to joints with knee injuries (juvenile injury group).

F: female; M: male; Na: not applicable; SF: synovial fluid; P: plasma; U: urine; RF-: negative rheumatoid factor; NSAID: non-steroidal anti-inflammatory drugs.

Biomarker assays
Technical performance of biomarkers assays and study samples are shown in Supplementary Table S1.

COMP
COMP is a non-collagenous protein in the extracellular matrix, which function is to stabilise the collagen network. It is most abundant in cartilage, but has been detected also in tendons, meniscus and synovial membrane. Upon cartilage degradation, COMP is released into the synovial fluid as an early event, and can later be found in the circulation. Synovial fluid and plasma COMP levels were quantified with an ELISA based immunoassay (BioVendor, no. RD194080200) according to the manufacturer's instructions. The technical performance of the COMP assay for synovial fluid samples has been described previously (31). For this study, three different lot numbers of the BioVendor COMP assay were used. No differences in concentrations were observed for samples run on different lot numbers (data not shown).

C2C
C2C is a neoepitope of type II collagen, generated by collagenase cleavage. It is well studied in OA and RA and has been suggested to associate with OA progression. C2C levels in synovial fluid and plasma samples were assayed using the C2C ELISA kit (IBEX, no. 60-1001-001), and urine samples were assayed by the C2C-HUSA ELISA kit (IBEX, no. 60-1017); both kits according to the manufacturer's instructions. The technical performances of the C2C assay for synovial fluid samples (25) and of the C2C-HUSA ELISA for urine samples (32) have been described. The same lot numbers of the IBEX C2C assay were used for comparison of C2C levels in plasma (JIA *vs*. healthy references). For comparison of C2C levels in synovial fluid (JIA *vs*. juvenile knee injury), two different lot numbers of the C2C assay were used, and therefore we used a conversion factor of 1.93 (previously determined for these specific lot numbers (25)). The two lot numbers used for the analysis of plasma and synovial fluid C2C levels in JIA patients were incompatible, and due to the lack of a conversion factor between these batches, no comparison between these biofluids were done. Large lot variations of recorded levels have been observed previously for the IBEX C2C assay (25).

ARGS-aggrecan
Aggrecanase cleavage of aggrecan leads to the release of N-terminal ARGS fragments, which have been detected in OA samples and is an early key event in arthritis and joint injuries. ARGS-aggrecan was quantified using Meso Scale Discovery (MSD) platform as described (34). Briefly, synovial fluid and plasma samples were deglycosylated for 3 h at 37°C with chondroitinase ABC and keratanase. MSD microplates were coated with capture antibody specific towards the G1 and G2 globular domains of human aggrecan (anti-HABR, Invitrogen no. AHP0022) overnight and blocked with 1% BSA and 1% non-fat dry milk in PBST. After sample addition and washing, ARGS-aggrecan was detected by a biotinylated monoclonal antibody (MAb OA-1) specific for the ARGS neoepitope and incubated with sulfo-tagged streptavidin (MSD) before analysed in a Sector Imager 6000 (MSD). For this study, two different lot numbers of AHP0022 were used, and for samples run on both lot numbers no difference in concentrations were observed (data not shown).

NTX-I
In bone, the most abundant matrix protein is collagen type I, and degradation products from collagen type I can be used to assess changes in bone resorption. Cross linked N-terminal collagen (NTX-I) are peptides derived from the degradation of type I collagen. NTX-I concentrations in plasma were determined by immunoassays (Osteomark NTx serum, no. 9021) and in urine (Osteomark NTx urine, no. 9006) according to the manufacturer's instructions. For this study, two different lot numbers of the Osteomark NTx serum assay were used, although for samples run on both lot numbers no difference in concentrations were observed (data not shown).

TRAP5b
TRAP5b is an enzyme expressed by bone resorbing osteoclasts which is used as a marker of osteoclast activation. The TRAP5b immunoassay (MicroVue TRAP5b, Quidel no. 8036), was developed for serum and plasma and the technical performance in synovial fluid has not previously been reported. Thus, an investigation of the technical performance in synovial fluid was conducted (Suppl. Table S1). The dilution linearity and spiking recovery of synovial fluid samples was determined in two randomly chosen samples from the juvenile injury cohort and the assay using synovial fluid behaved similar to serum and plasma samples reported by the manufacturer. The TRAP5b assay was run according to the manufacturers' instructions. For this study, two different lot numbers of the MicroVue TRAP5b assay were used, and for samples run on both lot numbers no difference in concentrations were observed (data not shown).

Ethics
The study is in accordance with the Helsinki Declaration, and was approved by the North Ethical Committee in Stockholm, Sweden and the regional ethical review board, Lund, Sweden. For the JIA and healthy reference samples, informed consent was given by the subjects and their parents. Knee injury patients were identified during the recruitment process (in Skåne region) of a longitudinal cohort study (ISRCTN 84752559, http://www.controlled-trials.com) in which these patients were not eligible for inclusion in that trial; consent to participate was given by the subjects.
Samples from this knee injury cohort have been used in several previous studies (25, 28, 31, 34, 35).

Statistics
Based on test for normality (Shapiro-Wilk, histogram and Q-Q plots) of the residuals, all models (*i.e*. ANCOVA test with adjustments for age, sex and number of affected joints) except synovial fluid ARGS and plasma COMP were normally distributed. When log10 transformed synovial fluid ARGS and plasma COMP concentrations were

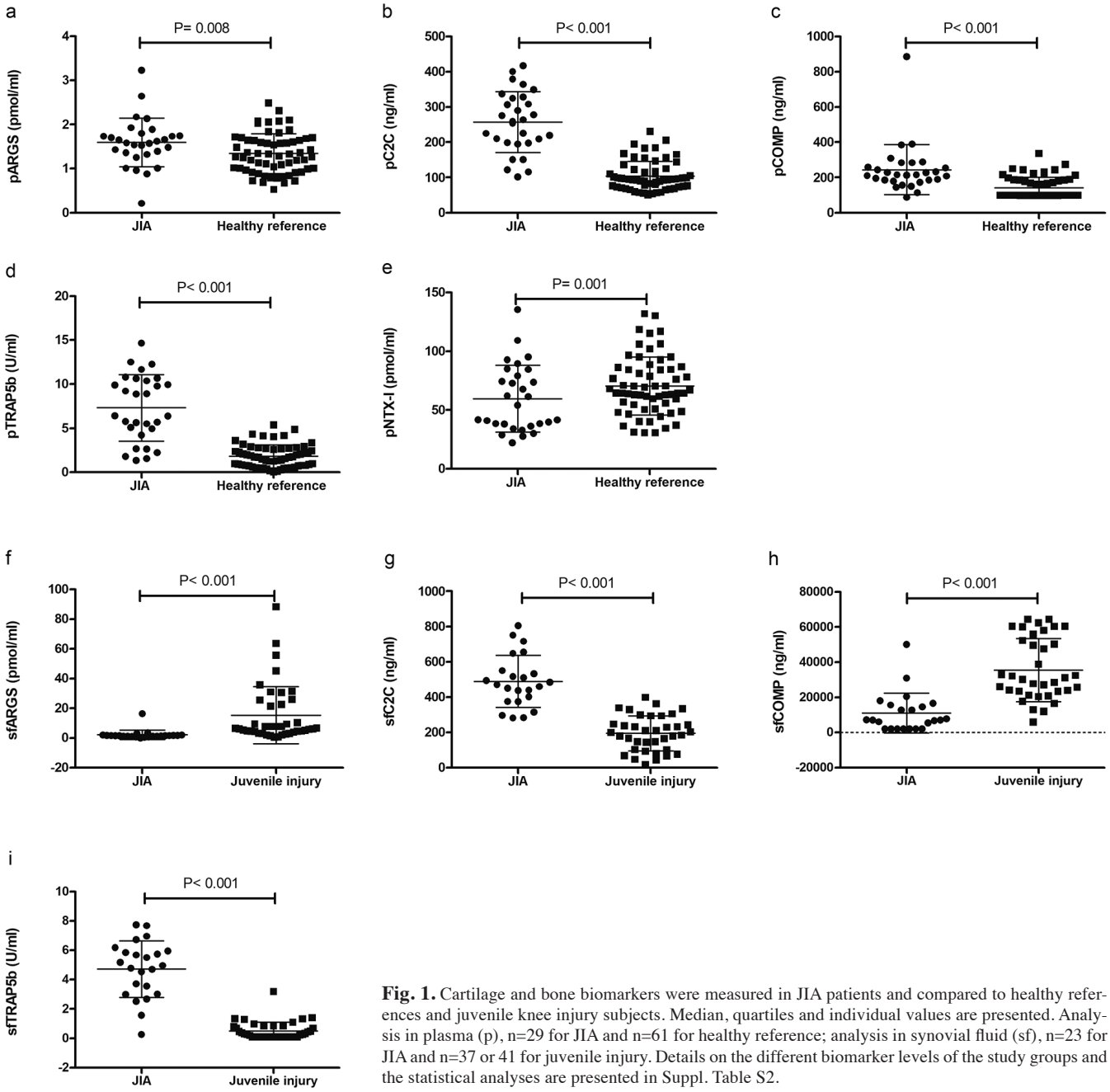

Fig. 1. Cartilage and bone biomarkers were measured in JIA patients and compared to healthy references and juvenile knee injury subjects. Median, quartiles and individual values are presented. Analysis in plasma (p), n=29 for JIA and n=61 for healthy reference; analysis in synovial fluid (sf), n=23 for JIA and n=37 or 41 for juvenile injury. Details on the different biomarker levels of the study groups and the statistical analyses are presented in Suppl. Table S2.

used, the residuals of these models were normally distributed. The actual values of concentration of synovial fluid C2C, and plasma ARGS and TRAP, and urine NTX-I were normally distributed, while the rest of the biomarkers were not. Therefore, for the non-adjusted model of biomarker comparison between subject groups, both non-parametric Mann-Whitney tests and parametric Student's *t*-test were used. Some of the samples had values below lower limit of detection (LLOD) presented in Suppl. Table S2, and for these, the values were imputed (equal to half the value of LLOD). In the correlation analyses between biomarkers (*i.e.* for the JIA group), no values were below LLOD; not all biomarkers were normally distributed, and therefore Spearman's rank order correlations were used. For comparison of biomarker concentrations in synovial fluid and plasma within JIA patients, non-parametric Wilcoxon signed ranks test were used. Differences in age between JIA and the other groups were analysed by Student's *t*-test, while differences in sex was analysed by Chi square test (none of the cells had expected count <5%). For the analyses for confounders in the JIA group, due to low n, non-parametric Mann-Whitney tests and Spearman's rank order correlations were used. Receiver operator characteristics (ROCs) analyses were used to evaluate whether biomarker concentrations could distinguish subjects with JIA compared to healthy references or juvenile injury. Evaluations were based on the area under the ROC curve (AUC) with cut-offs chosen to maximise the sum of the sensitivity and specificity.

Due to low sample volumes of plasma, healthy control subjects (n=193) were divided into three age- and sex-matched subgroups, n=61 each (Table I); single values per subject were analysed and between 1 and 3 biomarker analyses were done from each subject sample. For biomarker analysis using samples from JIA and juvenile knee injury subjects, mean values were calculated from duplicate values.

For statistical analysis, we used IBM SPSS Statistic (v. 24). All analyses were performed using 2-tailed tests and *p*-values less than 0.05 was considered statistically significant. We did not compensate for multiple testing due to the exploratory nature of the study. If not otherwise specified, expressions such as "higher" and "lower" in the text are based on statistically significant differences.

Results

Comparison of cartilage and bone biomarkers between the JIA group and reference or juvenile knee injury groups

Biomarker concentrations in the different study groups are presented in Suppl. Table S2. ANCOVA analyses (with adjustments for age, sex and number of affected joints) were used in the comparisons of biomarker concentrations between the JIA patients and the healthy reference or the juvenile knee injury groups. Compared to healthy references, the plasma concentrations of the cartilage biomarkers ARGS-aggrecan, C2C and COMP were between 1.2 and 2.4-fold higher in the JIA group. Similarly, the plasma concentrations of the TRAP5b bone biomarker was 4-fold higher in the JIA group, while plasma NTX-I levels were 1.2-fold lower (Fig. 1a-e, Suppl. Table S2).

Compared to juvenile knee-injured patients, the synovial fluid concentrations of the cartilage biomarkers ARGS-aggrecan and COMP and were 7.0 and 3.2-fold lower in the JIA group, while the cartilage C2C and the bone TRAP5b markers were 2.5 and 9-fold higher (Fig. 1f-i, Suppl. Table S2). ROC analyses revealed that the plasma concentrations of C2C and TRAP5b could differentiate JIA from healthy references with an AUC of 0.95 and 0.92, respectively, while ARGS, COMP and NTX-I had AUC between 0.63 and 0.82 (Suppl. Table S3). Synovial fluid concentration of C2C and TRAP5b could also separate JIA from juvenile injury, AUC of 0.96 and 0.97, respectively (Suppl. Table S3).

Association between biofluids in JIA patients

For the cartilage biomarker ARGS-aggrecan, no difference in concentrations between synovial fluid and plasma was detected, and the levels between biofluids did not correlate (Fig. 2a, Table II). In contrast, for the cartilage biomarker COMP the median of the synovial fluid level was 33-fold higher compared to the plasma level, although the levels measured in the two biofluids did not correlate (Fig. 2b, Table II). C2C levels in synovial fluid and plasma were recorded using plates from two different lot numbers and showed too large inter-batch variation to be compared. For the bone biomarker TRAP5b, the plasma levels were marginally higher (1.3-fold based on median) compared to the synovial fluid levels, and the levels in the two biofluids showed positive correlation (Fig. 2c, Table II). While none of the cartilage biomarkers correlated between biofluids, the two bone biomarkers, TRAP5b and NTX-I, showed strong positive correlations in plasma, and in plasma *vs*. urine (Fig. 2d, e, Table II).

Confounding factors for the biomarker concentrations in JIA patients

The age at sampling of JIA patients inversely correlated with plasma and urine levels of C2C and with synovial fluid and plasma levels of TRAP5b; no other biomarker correlated with age for these patients and none of the biomarkers correlated with disease duration (Table III). Female JIA patients had a median level of 1.4-fold higher plasma C2C and 1.3-fold higher plasma COMP levels than the male patients; no other differences in biomarkers levels between sexes were found (Table IV). Regarding clinical findings in this cohort, we investigated any effect on biomarker levels from number of affected joints at time of sampling, disease duration at time of sampling and if the patients were subjected to treatment. None of the biomarkers correlated with disease duration. Patients with two or more affected joints at time of sampling had 1.9-fold higher concentration of urine C2C; no other differences in biomarker levels were observed when comparing subjects with one affected joint *versus* several affected joints and no correlation was found between number of affected joints and biomarker concentrations (Tables III-IV). Also, no difference in biomarker concentrations were found between JIA patients under medication treatments compared to untreated patients (Table I and III).

Discussion

Children with JIA are at risk of developing permanent joint damage due to inflammation and degradation of cartilage and bone. By the time destruction is detectable with x-ray, the clinically used method of detection, joint damage may already have caused life-long disability. Biomarkers of cartilage and bone degradation have the potential to function as more sensitive or complementing tools to x-ray when detecting and evaluating destruction, which would be a desirable addition to the today available means. When evaluating the usefulness of biomarkers for disease-associated biological processes, it is important to characterise them in relation to confounding factors that may influence their levels.

In this exploratory study we have screened a set of biomarkers for cartilage and bone degradation in JIA patients, healthy children and in juveniles with knee injuries. All biomarkers were detectable in all sample types in our juvenile cohorts. For the JIA patients, we also examined the impact of possible confounding factors that could influence the levels of degradation products and compared levels of the different biomarkers measured in triplet biofluids (plasma, synovial fluid and urine) collected at the same time point.

We detected increased levels of the cartilage markers ARGS-aggrecan, C2C and COMP in plasma of JIA patients compared to healthy children, which implies a higher degree of cartilage

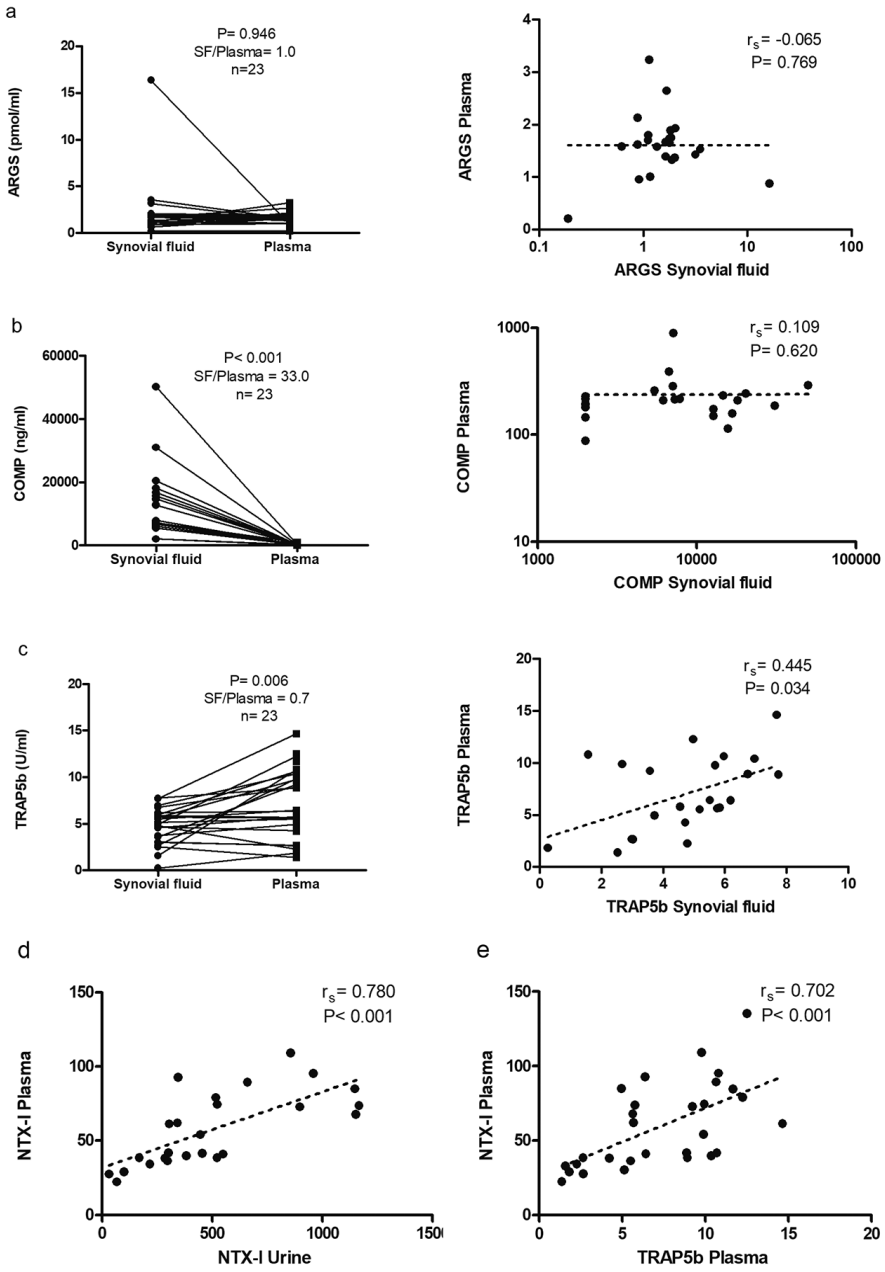

Fig. 2. Comparison of cartilage and bone biomarkers in different biofluids from JIA patients. Differences between biomarker biofluid concentrations were assessed by Wilcoxon analysis and correlation by Spearman's test (r_S). The ratio between synovial fluid (sf) and plasma (p) are based on median values. (**a**) ARGS-aggrecan, synovial fluid *vs*. plasma; note: x-axis in the right panel is in log 10 scale. (**b**) COMP synovial fluid *vs*. plasma; note: y-axis in the right panel is in log 10 scale. (**c**) TRAP5b, synovial fluid *vs*. plasma. (**d**) NTX-I, plasma vs urine (u). (**e**) Plasma NTX-I *vs*. plasma TRAP5b. Dotted lines = linear regression lines between biofluids.

degradation in JIA. Based on the AUC analyses, of the cartilage biomarkers C2C was better to distinguish JIA from healthy controls compared to ARGS-aggrecan and COMP. When comparing local (synovial fluid) between JIA and juveniles with knee injuries, we observed lower levels of ARGS-aggrecan and COMP in JIA patients, while increased levels of collagen C2C were recorded. We did not observe correlation between local and systemic levels for any of the cartilage markers, which is in contrast to previous studies in adult knee injuries, where synovial fluid and serum levels of C2C correlated (25). The absence of correlation between local and systemic levels of cartilage markers for the JIA patients found in this study could be due to contribution to systemic levels from other joints than the one where synovial fluid was aspirated. The JIA patients had up to 10 joints affected by arthritis at time of sampling (median 2 affected joints). However, no correlation was identified between number of active joints and level of any biomarker, nor when separating the patients as one joint affected compared to two joints or more.

Surprisingly, JIA patients had slightly decreased levels of the plasma bone marker NTX-I compared to healthy references. In contrast, it has been reported that serum levels of a similar bone marker, C-terminal type I collagen cross-linked telopeptide (CTX-I) was elevated in JIA patients compared to healthy children (36). However, the same study reported that in joints with higher degree of destruction, the serum concentration of CTX-I was significantly reduced. Our data may suggest a similar decrease in NTX-I marker levels in the JIA group, though this finding needs to be verified in a larger cohort. Concurrently, plasma levels of TRAP5b was elevated for the JIA group compared to healthy references and the synovial fluid levels were increased compared to juvenile knee injuries. Age dependent fluctuating serum levels of TRAP5b has been observed during normal bone growth in children (37), and we found in this study a negative correlation between plasma TRAP5b and age. Interestingly, we found a positive correlation between plasma and synovial fluid levels of TRAP5b as well as between plasma and urine levels of NTX-I. Plasma NTX-I and TRAP5b were also strongly correlated. TRAP5b showed both high sensitivity and specificity in distinguishing JIA from healthy references. Compared to assessments in synovial fluid, these data suggest that systemic assessments are good alternatives to monitor bone degradation in JIA.

We have previously shown that ARGS-aggrecan levels in synovial fluid from JIA patients are low compared to levels in children with knee injuries (20), and the low ARGS-aggrecan levels observed in JIA was confirmed in this study. These results may indicate either a lower aggrecan turnover in JIA

Table II. Correlation matrix of the biomarkers for the JIA group.

	sfC2C	sfCOMP	sfTRAP5b	pARGS	pC2C	pCOMP	pTRAP5b	pNTX-I	uC2C	uNTX-I
sfARGS	0.131 (0.553)	0.351 (0.100)	0.206 (0.345)	-0.065 (0.769)	0.169 0.442	0.045 (0.838)	-0.062 (0.778)	-0.206 (0.346)	-0.016 (0.943)	0.155 (0.481)
sfC2C		0.303 (0.160)	**0.461 (0.027)**	0.323 (0.133)	0.172 (0.433)	0.151 (0.492)	0.011 (0.961)	0.113 (0.609)	0.071 (0.747)	0.054 (0.805)
sfCOMP			-0.042 (0.849)	-0.041 (0.852)	0.052 (0.817)	0.109 (0.620)	-0.229 (0.292)	0.112 (0.610)	-0.072 (0.744	0.117 (0.594)
sfTRAP5b				0.246 (0.258)	0.413 (0.050)	0.145 (0.510)	**0.445 (0.034)**	0.247 (0.256)	0.124 (0.574)	0.195 (0.373)
pARGS					0.072 (0.710)	0.040 (0.836)	**0.600 (0.001)**	**0.552 (0.002)**	-0.019 (0.927)	0.206 (0.323)
pC2C						0.082 (0.674)	0.324 (0.086)	0.166 (0.391)	0.060 (0.776)	0.328 (0.109)
pCOMP		No sig.					0.157 (0.416)	0.193 (0.316)	-0.178 (0.395)	-0.107 (0.611
pTRAP5b		> 0.4						**0.702 (<0.001)**	0.228 (0.272)	**0.465 (0.019)**
pNTX-I		> 0.5							-0.155 (0.458)	**0.780 (<0.001)**
uC2C		> 0.6								-0.108 (0.606)

Spearman's rank order correlation coefficient with *p*-values in parentheses.
n=23 for synovial fluid (sf) biomarkers, and n: 25 or 29 for plasma (p) and urine (u) biomarkers.

Table III. Correlation between biomarkers and confounders for the JIA group.

	Age	Duration	Number of joints
sfARGS	-0.389 (0.066)	-0.360 (0.091)	0.074 (0.739)
pARGS	-0.280 (0.141)	-0.107 (0.580)	-0.185 (0.337)
sfC2C	0.079 (0.721)	-0.216 (0.322)	0.061 (0.782)
pC2C	**-0.537 (0.003)**	0.163 (0.398)	0.228 (0.235)
uC2C-HUSA	**-0.400 (0.047)**	-0.108 (0.608)	0.354 (0.083)
sfCOMP	0.125 (0.570)	-0.180 (0.411)	0.148 (0.501)
pCOMP	-0.044 (0.821)	0.251 (0.189)	0.137 (0.478)
sfTRAP5b	**-0.507 (0.014)**	0.043 (0.847)	-0.096 (0.664)
pTRAP5b	**-0.510 (0.005)**	0.139 (0.473)	0.064 (0.743)
pNTX-I	-0.203 (0.290)	0.159 (0.411)	0.092 (0.636)
uNTX-I	-0.279 (0.177)	0.026 (0.901)	0.073 (0.729)

Association of biomarker concentrations in synovial fluid (n=23), plasma (n=29) and urine (n=25) samples from JIA patients with age at sampling, disease duration and number of affected joints. The results are expressed as Spearman's rho with *p*-values in parentheses. Details on the characteristics of the subjects are presented in Table I. Significance, $p<0.05$, are printed in bold.
Sf: synovial fluid; p: plasma; u: urine.

patients or that JIA patients show a different degradation pattern of aggrecan, where a different set of enzymes are activated in JIA as compared to knee injuries of juveniles (20, 38). Systemic COMP has been suggested as a prognostic marker of joint destruction in RA. In JIA, COMP has been reported to be reduced compared to healthy references (17, 18, 39), which was not supported by our data in this study. Our results indicate that the tissue degradation in JIA differs from knee injuries, with increased osteoclast activation and degradation of collagen in JIA as opposed to higher aggrecan and COMP degradation in juveniles with knee injuries. This indicates different molecular signatures between these patient groups. Disease activity, measured as affected joints, disease duration and medication did not seem to influence the levels of biomarkers. This suggests that the differences in levels noted most probably are related to pathogenesis, rather than disease activity or duration. It is of course preferable if the degradation markers are possible to detect early in the disease course, or as a response to therapy, however this issue could not be addressed in our cross sectional study.

It is important to characterise molecular biomarkers in JIA patients in relation to confounding factors that may influence their levels. This is indeed central to determine in the juvenile population as children are growing which is known to affect the bone and cartilage turnover.

Limitations of this study include the unknown the status of joint destruction and a small study population and hence it should be considered hypothesis-generating and explorative. Due to the small sample size of the JIA group, we did not analyse JIA subtypes or gender separately, although this could be done in larger cohorts. In the group comparisons (JIA *vs*. healthy and JIA *vs*. injury), we adjusted for age, sex and number of affected joints.

There was no specific sampling time, which due to circadian rhythm might have affected some biomarker level recordings. However, for translation into clinical settings, it is preferable to define robust biomarkers independent of specific sampling times. The study would also have benefitted from comparing JIA patients with longitudinal follow-up. Due to small sample volumes available from the healthy reference subjects, single values from each biomarker was obtained leaving a higher degree of uncertainty of the values compared to the JIA and juvenile knee injury groups, where samples were run in duplicates. This was compensated by a larger number of samples (n=61) compared to the other groups (Table I). The strength of the study is that our cohorts are clinically well described and the access to triple-paired samples for the JIA group.

Conclusions

In this study, we have shown that JIA patients have elevated levels of bone and cartilage destruction biomarkers compared to healthy children, indicating a potential to serve as clinical prognostic or diagnostic tools for de-

Table IV. Biomarker concentrations and confounders for the JIA group.

ARGS		**Synovial fluid**				**Plasma**		
	n	**Median, (SD); pmol/ml**	**Norm**	***p*-values**	**n**	**Median, (SD); pmol/ml**	**Norm**	***p*-values**
Sex								
Female	12	1.67 (0.83)	1	-	18	1.56 (0.52)	1	-
Male	11	1.65 (4.50)	0.99	0.496	11	1.62 (0.60)	1.04	0.405
Affected joints								
One joint	12	1.67 (0.73)	1	-	12	1.70 (0.73)	1	-
Two or more joints	11	1.65 (4.54)	0.99	0.916	17	1.53 (0.35)	0.90	0.091
Medication								
Yes	9	1.65 (0.94)	1	-	13	1.57 (0.46)	1	-
No	14	1.74 (4.01)	1.05	0.526	16	1.66 (0.61)	1.07	0.417
C2C		**Synovial fluid**				**Plasma**		
	n	**Median, (SD); ng/ml**	**Norm**	***p*-values**	**n**	**Median, (SD); ng/ml**	**Norm**	***p*-values**
Sex								
Female	12	253.87 (60.08)	1	-	18	284.22 (81.81)	1	-
Male	11	238.81 (92.19)	0.94	0.833	11	209.48 (82.01)	0.74	**0.049**
Affected joints								
One joint	12	239.36 (93.62)	1	-	12	232.23 (76.42)	1	-
Two or more joints	11	244.62 (55.45)	1.02	0.608	17	278.06 (91.57)	1.20	0.283
Medication								
Yes	9	251.43 (62.20)	1	-	13	263.78 (72.86)	1	-
No	14	241.72 (84.57)	0.96	0.781	16	217.77 (94.50)	0.83	0.249
COMP		**Synovial fluid**				**Plasma**		
	n	**Median, (SD); µg/ml**	**Norm**	***p*-values**	**n**	**Median, (SD); ng/ml**	**Norm**	***p*-values**
Sex								
Female	12	7.11 (8.84)	1	-	18	231.15 (165.53)	1	-
Male	11	12.74 (13.70)	1.79	0.560	11	179.30 (76.44)	0.78	**0.040**
Affected joints								
One joint	12	6.60 (6.79)	1	-	12	213.30 (197.00)	1	-
Two or more joints	11	7.82 (14.73)	1.19	0.442	17	216.50 (85.27)	1.02	0.957
Medication								
Yes	9	7.07 (9.14)	1	-	13	228.40 (68.94)	1	-
No	14	7.21 (12.92)	1.02	0.975	16	211.35 (181.64)	0.93	0.523
TRAP5b		**Synovial fluid**				**Plasma**		
	n	**Median, (SD); U/ml**	**Norm**	***p*-values**	**n**	**Median, (SD); U/ml**	**Norm**	***p*-values**
Sex								
Female	12	5.46 (2.17)	1	-	18	6.08 (4.07)	1	-
Male	11	4.78 (1.73)	0.88	0.833	11	8.88 (3.38)	1.46	0.877
Affected joints								
One joint	12	4.88 (1.93)	1	-	12	9.35 (4.06)	1	-
Two or more joints	11	5.18 (2.02)	1.06	0.928	17	5.69 (3.58)	0.61	0.370
Medication								
Yes	9	5.84 (1.73)	1	-	13	8.88 (3.72)	1	-
No	14	4.75 (2.01)	0.81	0.141	16	6.11 (3.91)	0.69	0.619
NTX-I		**Plasma**				**Urine**		
	n	**Median, (SD); pmol/ml**	**Norm**	***p*-values**	**n**	**Median, (SD); pmol//ml**	**Norm**	***p*-values**
Sex								
Female	18	57.90 (29.77)	1	-	14	344.02 (349.56)	1	-
Male	11	41.51 (27.48)	0.72	0.580	11	522.34 (324.18)	1.52	0.267
Affected joints								
One joint	12	57.90 (27.48)	1	-	12	415.15 (339.46)	1	-
Two or more joints	17	41.66 (29.83)	0.72	0.616	13	455.10 (356.45)	1.10	0.894
Medication								
Yes	13	62.07 (31.12)	1	-	10	364.67 (338.94)	1	-
No	16	47.66 (26.67)	0.77	0.503	15	516.09 (352.92)	1.42	0.765
C2C-HUSA		**Urine**						
	n	**Median, (SD); pg/ml**	**Norm**	***p*-values**				
Sex								
Female	14	293.21 (522.26)	1	-				
Male	11	158.84 (808.14)	0.54	0.075				
Affected joints								
One joint	12	159.63 (460.59)	1	-				
Two or more joints	13	305.87 (774.61)	1.92	**0.030**				
Medication								
Yes	10	347.87 (929.22)	1	-				
No	15	239.36 (227.96)	0.69	0.196				

Biomarker concentrations in synovial fluid, plasma and urine samples from JIA patients are stratified into the confounder groups of sex, number of affected joints and medication. For group comparisons Mann-Whitney tests were used. Details on the characteristics of the subjects are presented in Table I.
Norm: normalised median values. Significance, $p<0.05$, are printed in bold.

structive joint disease. In particular, assessments of C2C and TRAP5b in plasma samples suggest the possibility to distinguish JIA from healthy with high specificity and sensitivity. For the JIA group, gender, disease duration, number of affected joints and medication did not affect biomarker levels in this study; bone markers displayed good correlation of local and systemic levels, while cartilage markers showed no or low correlation between levels of synovial fluid and plasma; hence, bone markers appear suitable for systemic measurement. Moreover, our data indicate a molecular signature of destruction for JIA, which gives us important clues about the destructive process during disease progression. Future studies should focus on the correlation between radiologic imaging and clinically verified destruction with these markers to scrutinise their potential.

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