EXTENDED REPORT

Assessment of the clinical significance of gelatinase activity in patients with juvenile idiopathic arthritis using quantitative protein substrate zymography

N J Peake, H E Foster, K Khawaja, T E Cawston, A D Rowan

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See end of article for authors' affiliations

Correspondence to:
Dr A D Rowan,
Musculoskeletal Research
Group, School of Clinical
Medical Sciences, Medical
School Cookson Building,
University of Newcastle
upon Tyne, NE2 4HH, UK;
a.d.rowan@ncl.ac.uk

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Objective: To measure gelatinase activities in paired synovial fluid (SF) and serum of patients with juvenile idiopathic arthritis (JIA), and to assess how these activities relate to clinical and laboratory measures of disease activity.

Methods: A quantitative protein substrate zymography method was adapted and validated for use with serum and SF. Bands of activity were measured by densitometry and correlated with standard laboratory indicators of inflammation: erythrocyte sedimentation rate and platelet count.

Results: Gelatinase activity was found consistently in patients with JIA, with reproducible, quantified bands of activity corresponding to pro-matrix metalloproteinase-9 (pro-MMP-9), including the neutrophil associated lipocalin complex, and pro- and active forms of MMP-2. Both active MMP-2 and pro-MMP-9 were higher in JIA serum than in controls, though no differences were seen between patients grouped according to age, disease duration, or JIA subtype. However, SF MMP-9 correlated significantly with the laboratory indicators of inflammation, as did the relative level of active MMP-2.

Conclusions: Both MMP-2 and MMP-9 gelatinolytic activities are raised during active JIA and associated with inflammatory activity regardless of age and disease duration, supporting a role for MMPs in the breakdown of joint components from early in disease. These MMPs may be specific markers of active joint destruction linked to inflammatory JIA, MMP-9 as a product of infiltrating cells, and the activation of MMP-2 produced within the joint.

The matrix metalloproteinases (MMPs) are a family of neutral, zinc dependent endopeptidases currently consisting of 23 members in humans,¹ and are heavily implicated in the joint breakdown seen as a consequence of chronic arthritic disease. Collectively, they can degrade most components of cartilage and are up regulated by proinflammatory cytokines present in the joint compartment during chronic inflammation.² They can be detected in synovial fluid (SF) and immunolocalised to the synovial membrane and cartilage from diseased joints,^{3–4} and are produced as inactive zymogens, which are activated in physiological conditions by a cascade of proteolytic reactions, resulting in cleavage of a 10 kDa pro-region of the enzyme.⁵

Protein substrate zymography is a useful technique for the sensitive detection of MMPs, and has been successfully employed in the identification of enzyme activity in the SF of arthritic joints in both humans and animals.^{6–7} Gelatin zymography can discriminate between MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in both pro- and active forms without interference from specific and non-specific inhibitors present in human serum (and SF) because of size dependent separation through an acrylamide gel. The pro-forms of MMPs are activated after the denaturation/renaturation that occurs in the assay through treatment with sodium dodecyl sulphate (SDS).⁵ MMP-2 is found at a molecular weight of 72 kDa in its pro-form, and 66 kDa in the active form. It is of considerable relevance to chronic arthritic disease because it has been shown to have weak activity against type II collagen, as well as efficient activity against denatured collagen (gelatin) and other substrates of the cartilage matrix.⁸ Denaturation and degradation of type II collagen are considered to be key events in irreversible joint damage, where the triple helical collagen molecule is cleaved across all three polypeptide chains.⁹

By contrast, the larger MMP-9 protein has a molecular weight of 94 kDa in the pro-form, and 82 kDa in the active form. However, MMP-9 isolated from neutrophils appears to separate into three bands of activity,¹⁰ corresponding to the monomeric pro-form, a high molecular weight (>180 kDa) dimeric pro-form,¹¹ and a 110 kDa complex associated with the lipocalin protein, which may have a role in activation of MMPs.¹²

The term juvenile idiopathic arthritis (JIA) covers a heterogeneous group of diseases with an age of onset under 16, with variable outcome and response to treatment. The Durban classification, refined in 2004,¹³ identifies seven clinically distinct subgroups of JIA. The more common subtypes are defined according to the number of joints affected within the first 6 months. In polyarticular patients, who can be rheumatoid factor positive or negative, more than four joints are affected within 6 months, whereas in oligoarticular patients it is four or less. However, 50% of oligoarticular patients develop an extended phenotype, progressing to a condition in which more than four joints are affected after the 6 months. The remaining subtypes, systemic, enthesitis related, and psoriatic related JIA, are diagnosed according to extra-articular manifestations. At present, the cause of JIA and its heterogeneity are unknown, and therefore diagnosis is made by exclusion. Long term outcome studies have shown that, contrary to early reports, many patients have active disease and considerable morbidity

Abbreviations: DMARD, disease modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; FRH, Freeman Hospital; ILAR, International League Against Rheumatism; JIA, juvenile idiopathic arthritis; MMP, matrix metalloproteinase; NSAID, non-steroidal anti-inflammatory drug; PLT, platelet count; SDS, sodium dodecyl sulphate; SF, synovial fluid

into adulthood,¹⁴ and recent radiographic studies indicate that joint damage is thought to occur earlier than previously believed.¹⁵

Little direct data exist on the activity of proteinases during active JIA despite the radiographic evidence of early damage, and the potential effects of disease in developing cartilage which is structurally distinct from older tissue.^{16–17} MMP-3 (stromelysin-1) has been measured in JIA SF,¹⁸ and is present at very high levels. Stromelysins have a broad substrate range, and are implicated in proteoglycan breakdown¹⁹ and activation of other MMPs.^{20–21} MMP-3 appears to diffuse from the joint into serum, where it is also measurable,²² and correlates significantly with disease activity. Perhaps importantly, in a co-culture model of osteoarthritic articular cartilage chondrocytes and macrophages, MMP-9 was produced by macrophages and activated by MMP-3 produced by the chondrocytes in response to macrophage infiltration.²³ However, there is also evidence that MMP-9 is produced by chondrocytes and fibroblasts within the joint during disease.^{24–25}

Protein substrate zymography has been shown to be quantifiable and reproducible for assaying the SF of patients with osteoarthritis, and gelatinase activity appeared to reflect the degree of erosive damage in these patients.²⁶ This study aimed at investigating gelatinases in paired JIA serum and SF samples using a zymography technique capable of discriminating between pro- and active-MMPs on the basis of size, and investigating whether their presence and activation was associated with inflammatory activity using standard laboratory and clinical measures of disease. We proposed the hypothesis that the presence of MMPs linked to inflammation from an early point in disease might contribute to the early joint damage seen in JIA, and therefore used a patient group which included young patients with a recent diagnosis through to adults with decades of active disease.

PATIENTS AND METHODS

Patients and samples

The study was performed on patients with JIA who donated paired serum and SF samples between 1996 and 2003 to a sample bank at the Freeman hospital (FRH), Newcastle upon Tyne. The patients included those with early onset disease up to adults with several decades of disease tracked through the FRH transitional services. Parental or patient consent was obtained, and sample collection performed under appropriate ethical guidelines. Fifty patients were selected, all diagnosed using the International League Against Rheumatism (ILAR) criteria,¹³ of whom 31 (62%) were female. The ILAR subtype of the patients was recorded as polyarticular JIA (*n* = 10) or oligoarticular JIA (*n* = 40); 11 of the latter had extended disease.

Serum and SF samples were obtained at the time of routine joint aspiration procedures, and centrifuged at 1300 *g*; 10 minutes for serum, and 30 minutes for SF. The supernatants were divided into aliquots and stored at –80°C until

use. A total of 82 paired SF and serum samples were available from the patients selected. Retrospective analysis of patient notes was performed to obtain the routine laboratory data used to monitor the level of inflammation and response to treatment at the time of sampling where available.

Erythrocyte sedimentation rate (ESR) and blood platelet count (PLT) were recorded (for 72 (88%) and 63 (77%) sample points, respectively), along with basic clinical data; age of onset, disease duration, and age at study entry. Medication details were also recorded at these sample points (non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying antirheumatic drugs (DMARDs) or steroid treatment) in order to assess the impact of treatment on gelatinase levels. For controls, healthy laboratory volunteers and children admitted for non-inflammatory conditions were recruited for blood donation with ethical approval (*n* = 13). The mean age at sampling was 26 years (range 9–37), and six (46%) were female. Blood was collected in Vacuette EDTA tubes (for plasma), then half was removed into Vacuette serum tubes with clot activator (Greiner Bio-One, Gloucestershire, UK). Both blood samples were then centrifuged at 1300 *g* for 10 minutes and the supernatant removed and stored at –80°C until analysis.

Zymogram method

Zymography was performed by co-polymerising gelatin (BDH Chemical Co, Poole, UK) at a final concentration of 1 mg/ml in a 0.75 mm thick 7.5% acrylamide gel using Mini-protein II equipment (Bio-Rad Laboratories, Hertfordshire, UK) with a 4.5% acrylamide stacking gel. Samples (1 µl) were diluted 10-fold in loading buffer (4% glycerol, 1% (wt/vol) SDS, and 0.125% (wt/vol) bromophenol blue in 0.125 M Tris-HCl, pH 6.8). A SeeBlue molecular weight ladder (Invitrogen Ltd, Paisley, UK) was used to enable size determination of the activity bands. Electrophoresis was performed at 200 V/40 mA for each gel in 25 mM Tris, 0.2 M glycine, and 1% (wt/vol) SDS running buffer, pH 8.2, until the tracking dye reached the bottom of the gel. Gels were then transferred to a 100 mm² Petri dish, rinsed in water, then washed twice with 50 ml 2.5% (vol/vol) Triton X-100 (Sigma Chemical Co, Poole, UK) in 20 mM Tris-HCl, pH 7.8, with agitation at room temperature (30 minutes for each wash). The gels were finally rinsed three times in water, and then incubated for 20 hours in 50 ml of 1% (vol/vol) Triton X-100 in 20 mM Tris-HCl, pH 7.8, at 37°C.

Measurement

After incubation, the gels were rinsed with dH₂O and then stained with 2.5% (wt/vol) Coomassie R-250 (Sigma) in 40% (vol/vol) methanol, 10% (vol/vol) acetic acid for 1 hour at room temperature with agitation. The gels were then destained in 40% (vol/vol) methanol, 10% (vol/vol) acetic acid for 30 minutes, and bands of activity visualised as clear lysis zones on a blue background using a Chemigenious bioimaging system and GeneSnap gel documentation software (SynGene Ltd, Cambridge, UK). The images were then

Table 1 Clinical characteristics of the patients with JIA studied

Characteristics	Persistent oligoarthritis (<i>n</i> = 29)	Extended oligoarthritis (<i>n</i> = 11)	Polyarticular (<i>n</i> = 10)
Age of onset (years)	7 (1–16)	8 (1–16)	8 (1–16)
Disease duration at study entry (years)	9 (1–27)	6 (1–30)	8 (1–28)
Age at study entry (years)	16 (4–34)	14 (2–36)	16 (5–33)
Mean ESR (mm/1st h)	28 (2–55)	16 (1–79)	26 (2–83)
Mean PLT ($\times 10^9$ cells/l)	388 (216–599)	317 (191–448)	414 (294–636)

Data are presented as the mean value (range).

No significant differences were found between subtypes for any of the variables studied.

Figure 1 Validation of gelatin zymography of JIA SF and serum samples. (A) The use of quantitative zymography was validated by running test samples, and five distinct gelatinolytic activities were routinely observed in JIA SF and serum samples. (B) A standard curve was generated from human recombinant MMP-2 across a linear range of 0.05–1 ng/ml, and the pixel density in the middle of this range (0.5 ng/ml) used to calibrate the values obtained by densitometric analysis of the gelatinase activities in patient samples to generate arbitrary units (AU). (C) Running the test samples on different days using fresh reagents confirmed that the assay was a robust, reproducible way of assessing gelatinase activity, and showed clear dilution factor dependent changes in activity; total MMP-9 and total MMP-2 activity are illustrated for three SF and three serum samples (mean (SEM), $n=4$). Total MMPs were calculated as described in the text.

quantified at the same magnification using ImageMaster 1D software (Amersham Biosciences, Buckinghamshire, UK), and recorded as pixel density based on band intensity and size after rolling-disc background subtraction. The arbitrary densitometry units were defined by calibrating to a standard curve prepared from human purified recombinant MMP-2 kindly donated by Professor Gill Murphy (Department of Oncology, Cambridge Institute for Health Research, University of Cambridge, UK). In some experiments, the metalloproteinase inhibitor 1,10-phenanthroline (Sigma) was used at 2 mmol/l final concentration in the incubation buffer in order to confirm that the activities were due to MMPs.

Statistical analysis

Statistical analysis of the measured activities was performed by Spearman's rank correlation of continuous variables, and by the Mann-Whitney test for group comparisons when analysing two groups, and Kruskal-Wallis test (with Dunn's post hoc test) for comparisons of more than two groups.

Where multiple samples were available for a patient, mean values were used in the statistical analysis to ensure independence of the data points.

RESULTS

Patients and activity

Table 1 shows the clinical characteristics of the patients included in the study, split into the separate JIA subtypes diagnosed. No significant differences were seen between the subtypes for the clinical variables age of onset, disease duration or age at sample time. Neither were differences seen between the laboratory indicators of inflammation, ESR and PLT. Of the 82 samples obtained from the patients, 54 were taken during NSAID treatment, 19 during corticosteroid treatment (intravenous or oral), 28 during methotrexate treatment, and 3 during etanercept treatment.

Zymography validation

The gelatin zymography yielded five distinct gelatinase activities present in both SF and serum samples (fig 1A).

Figure 2 Comparison of gelatinase activity in JIA patient sera and SF with normal control serum and plasma. (A) Plots of the three pro-MMP-9 activities at 250 kDa, 110 kDa, and 95 kDa. These bands were significantly higher in control serum than in control plasma. Notably, JIA serum showed significantly higher 110 kDa lipocalin-complexed pro-MMP-9 activity than normal serum. SF activity was significantly lower than paired serum activity for the 250 kDa and 95 kDa bands. (B) Plots of the pro- and active MMP-2 bands seen at 70 kDa and 65 kDa, respectively, and the ratio of active:pro-MMP-2. The pro-MMP-2 activity at 70 kDa was significantly higher in control serum than in plasma, and JIA serum levels of pro-MMP-2 were not significantly higher than in control serum. Levels of both pro- and active MMP-2 were significantly higher in JIA SF than in paired serum, and notably, the level of active-MMP-2 was significantly higher in JIA serum than in control serum. This was also true of the active:pro-MMP-2 ratio, at a greater level of significance ($p<0.0001$). Selected statistical comparisons are shown: * $p<0.05$; ** $p<0.01$; *** $p<0.0001$, Kruskal-Wallis test (with Dunn's post hoc test).

Gelatinase activities at 250 kDa, 110 kDa, and 95 kDa corresponded to the molecular weights of neutrophil pro-MMP-9, and activity at 70 kDa and 65 kDa with the molecular weights of pro- and active MMP-2, respectively.¹⁰ The class of these enzymes was confirmed by the use of an MMP inhibitor, 1,10-phenanthroline, which completely abolished these activities (data not shown). A standard curve generated from a positive control of human recombinant MMP-2 was also run on the zymogram (fig 1B). The pixel

density corresponding to the middle of this curve, 1 064 147 pixels, was used to calibrate the sample values. The resulting arbitrary units (AU), standardised to 0.5 ng MMP-2, are used from here on.

The reproducibility of the quantitative zymography was confirmed by running test samples on different days using fresh reagents. Total pro-MMP-9 was calculated by summing the 250 kDa, 110 kDa, and 95 kDa activities, and total MMP-2 by summing the 70 kDa and 65 kDa activities. Figure 1C

Figure 3 SF gelatinase activity in patients with JIA grouped by disease duration. Three disease duration groups were defined, 0–2 years ($n=18$), 2–6 years ($n=16$), and 6–29 years ($n=16$); no significant differences were seen between the groups for MMP-9 (A), MMP-2 (B), or the active:pro-MMP-2 ratio (C). No significant differences were observed either for serum gelatinase activity.

Figure 4 Correlation of MMP-9 gelatinase activities with laboratory measures of inflammation. ESR and PLT determined at the point of sampling were correlated with the gelatinase activities corresponding to isoforms of pro-MMP-9. Significant Spearman's rank correlation values (r_s) were found for the 110 kDa lipocalin complexed pro-MMP-9 v ESR (A) and PLT (B), and for the 95 kDa activity v PLT (C), where * $p < 0.05$; ** $p < 0.01$.

shows reproducible sample dilution curves for total MMP-2 and total pro-MMP-9. The intra-assay variability of the technique was determined by running duplicate samples (10 duplicates, run in two independent experiments), and the coefficients of variation for the duplicates were 8–14% (mean 11.2%). Interassay variation was calculated from six samples repeated in four independent experiments, and the coefficients of variation were 5.7–16.4% (mean 9.4%).

Gelatinase activity in patients with JIA and control samples

The JIA samples chosen for analysis were analysed by densitometry of each of the five bands of activity and converted to arbitrary units. Values for each individual band, total MMP-2 and pro-MMP-9, and the ratio of active:pro-MMP-2 were analysed, and were not found to be significantly different between the subtypes in either serum or SF. However, it was noted that MMP-2 activity was significantly higher in SF than in paired sera ($p < 0.0001$ for both pro- and active forms), and pro-MMP-9 activity significantly higher in serum than in paired SF ($p < 0.0001$ for all isoforms, fig 2). We further observed that the 110 kDa lipocalin complexed pro-MMP-9 band, and the active MMP-2 band were higher in JIA patient serum than in control (fig 2). Finally, we also noted that all of the pro-MMP-9 activities and the 70 kDa pro-MMP-2 activity were higher in control serum than in paired plasma (fig 2). This was also true for the active:pro-MMP-2 ratio, and indicated that sample collection was an important consideration of the data.

The pro-MMP-9 activities were highly correlated with each other in both SF ($r_s = 0.8–0.9$, $p < 0.0001$), and in serum ($r_s = 0.7–0.8$, $p < 0.0001$). This would be expected if these isoforms are produced concordantly. In SF, they also correlated less strongly with the MMP-2 gelatinase activities

($r_s = 0.3–0.4$, $p < 0.05$), and the pro- and active-MMP-2 forms were found to correlate together in SF ($r_s = 0.3$, $p < 0.05$).

Relationship of gelatinase activity to clinical and laboratory measures

The role of gelatinases in JIA was investigated using the clinical and laboratory measures obtained at the time of sampling. No differences were seen in gelatinase activity when patients were grouped by NSAID, DMARD or corticosteroid treatment. Gelatinase activity did not correlate with age of onset, disease duration or age at study entry, and when patients were grouped according to disease duration at study entry, no significant differences were seen (fig 3). The gelatinase data were also correlated with the standard laboratory measures of inflammation in order to investigate whether gelatinase production and activation were linked with the inflammatory process. The 110 kDa lipocalin complex and the 95 kDa monomeric isoforms of pro-MMP-9, and active (65 kDa) MMP-2 in SF correlated significantly with ESR and PLT (MMP-9, fig 4; MMP-2, fig 5A). Furthermore, the ratio of active:pro-MMP-2 also correlated with ESR (fig 5B). Thus, pro-MMP-9 production was linked to inflammation, whereas MMP-2 production itself did not appear to be linked; rather, the activation of MMP-2 correlated significantly with inflammatory activity. It is noteworthy that no active MMP-9 was identified in these patients.

DISCUSSION

Although MMP levels have been extensively studied in the adult arthritides,^{1–4} relatively few studies to date have measured these potent proteinases in JIA. As a childhood onset disease that often persists into adulthood, JIA has severe implications as it leads to persistent up regulation of

Figure 5 Correlation of MMP-2 gelatinase activities with laboratory measures of inflammation. ESR and PLT determined at the point of sampling were correlated with the 70 kDa and 65 kDa gelatinase activities that are due to the pro- and active forms of MMP-2, respectively, as well as the ratio active:pro-MMP-2. Significant Spearman's rank correlation values (r_s) were found for the 65 kDa active MMP-2 activity (A) and the active:pro-ratio (B) v ESR, where ** $p < 0.01$.

the proteinases capable of degrading cartilage components; significantly, joint damage is now considered to occur earlier than previously thought in JIA.¹⁵ In addition to these unique clinical features, the structure of cartilage at different ages has been shown to differ considerably,^{16,17} and the implications for juvenile onset disease and susceptibility to MMP mediated joint damage are not known. MMP-3 has previously been found to be a good marker of disease activity,^{18,22} but there are currently no data available on collagenases or gelatinases in samples from patients with JIA.

As far as we know, this is the first report of enzymes capable of degrading gelatin (denatured collagen) in JIA, and we show that MMP-9 and MMP-2 are found in both SF and serum samples using gelatin substrate zymography. Of potential significance, MMP-2 also exhibits weak activity against type II collagen,⁸ which is considered to be important in irreversible joint damage²⁷; indeed, MMP-2 was found to correlate with collagen loss in soft connective tissue explants, even though the classical collagenases such as MMP-1 did not.²⁸

We showed that both MMP-2 and MMP-9 concentrations were higher in control serum than in control plasma samples. It has been demonstrated previously that sample collection is an important variable in determination of gelatinases by both enzyme linked immunosorbent assay (ELISA) and zymographic techniques,^{29,30} as the clotting procedure for sera results in activation of blood PLT and leucocytes, causing the release of MMP-2 and MMP-9. This may partially explain why JIA SF measurements showed significant correlations with laboratory indicators of inflammation, whereas serum measurements did not. MMP-2 is also produced by chondrocytes and fibroblasts,^{10,31} which probably contribute to its presence in SF during active inflammatory disease; this would account for the higher levels seen in SF compared with paired serum, even with the bias towards higher serum levels caused by sample clotting, given that its production is up regulated by the proinflammatory cytokines thought to drive joint destruction.² It is notable, however, that we did not observe a correlation of pro-MMP-2 with inflammatory activity as assessed by ESR and PLT, but did see a correlation between active MMP-2 and ESR; and even more significantly, for the ratio between pro- and active MMP-2. These data suggest that it is not increased production of this MMP that is necessarily most closely linked to inflammation (or that increased production occurs at a low inflammatory threshold during active disease), but enzyme activation—which is a necessary step for proteolytic activity. Many MMPs have been found to be produced in adult arthritic joints,⁴ and activation by other MMPs is a key step in cartilage breakdown.³² It is therefore clear that the proteolytic processes occurring in this destructive childhood onset disease require further study.

MMP-9 is produced by neutrophils and macrophages, but also by fibroblasts and chondrocytes within the joint, after proinflammatory stimuli.^{24,25} We propose, however, that neutrophils produce the majority of MMP-9 during active disease, owing to the presence of the 110 kDa neutrophil associated lipocalin complex. This isoform correlated highly with the other isoforms, suggesting it is produced along with the monomeric and dimeric latent forms of MMP-9. It is notable that no discernible MMP activity was found at 82 kDa, corresponding to active MMP-9, or indeed any activity corresponding to active dimeric MMP-9, which has a similar gelatinolytic activity but differs in the rate at which it is activated by MMP-3.¹¹ The ability of MMP-3 to activate MMP-9 (as well as other MMPs) is interesting as MMP-3 has been found to be present in large amounts in JIA SF. Although no active MMP-9 was seen in patient samples, it may be that activation is occurring at specific sites of joint

damage mediated by the large concentration of proteolytic activators present.

A weak gelatinase activity was seen in both serum and SF samples that was not inhibited by 1,10-phenanthroline, at around 80 kDa (data not shown). In the absence of the metalloproteinase bands, this activity appeared to be a non-metalloproteinase triplet. Although the identity of these activities is not known, a similar triplet of gelatinolytic activity has been previously reported in human serum, but not in SF.³³ Blood contamination of SF can be excluded in this study, however, because any contaminated samples are routinely discarded at the FRH clinical laboratory. This activity did not relate to any of the clinical and laboratory criteria during our analyses, and was therefore not studied further.

Casein can also be adapted as a substrate for protein zymography, and we developed a reproducible, quantitative casein zymogram suitable for use with JIA samples. However, many MMPs are caseinolytic and have molecular weights at 50–70 kDa, including MMP-1, -2, -3, -10, and -13. We identified caseinolytic metalloproteinase activities in the 50–70 kDa range in JIA samples, but were unable to identify the specific MMPs responsible, whether they were pro- or active, or whether they were caused by a single MMP or multiple enzymes. Further work would be useful to develop the zymography method as a means of assessing proteolytic activity in patients using multiple protein substrates.

In conclusion, both the presence of pro-MMP-9 activity and the activation of MMP-2 appeared to be linked to the level of disease activity in patients with JIA, causing raised serum levels in comparison with controls, and both MMP-2 and MMP-9 activities were found at similar levels regardless of disease duration and age. This is significant, as the presence of enzymes capable of degrading cartilage components such as native and denatured collagen corroborates the concept of joint damage occurring early in JIA. Targeted, aggressive treatment towards poor prognostic disease is now widely advocated, and there is considerable interest in developing anti-MMP therapeutics in the future.³⁴ Identification of the potential targets of such treatment is an essential step for the application of these principles to juvenile onset disease.

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Authors' affiliations

N J Peake, H E Foster, T E Cawston, A D Rowan, Musculoskeletal Research Group, School of Clinical Medical Sciences, University of Newcastle-upon-Tyne, UK
K Khawaja, Department of Paediatrics, Newcastle Hospitals NHS Trust, Newcastle-upon-Tyne, UK

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