Chapter 7

Matrix metalloproteinase 1 and 3 promoter polymorphism in patients with early rheumatoid arthritis


Submitted.
Chapter 7

SUMMARY

Objective. To evaluate the significance of matrix metalloproteinase-1 (MMP-1) and MMP-3 promoter polymorphism in relation to disease activity and radiological damage in patients with early rheumatoid arthritis (RA).

Methods. MMP-1 and MMP-3 promoter polymorphisms were determined in a cohort of 448 early RA patients. Clinical and laboratory markers of disease activity and severity at presentation were related to these polymorphisms. In addition total radiological damage and radiological progression (Sharp’s method, modified by van der Heijde) after 2 years were evaluated. In a subgroup initial serum MMP-3 (sMMP-3), cumulative sMMP-3 (serum MMP-3 area under the curve; sMMP-3\(^{AUC}\)) over 0.5 and 1 year and sMMP-3\(^{AUC}\) relative to CRP\(^{AUC}\) in relation to the MMP-3 promoter polymorphism were studied.

Results. An association between MMP-1 and MMP-3 promoter polymorphism and disease activity, severity or radiological damage at presentation was not found. MMP-1 and MMP-3 promoter polymorphisms showed strong linkage disequilibrium, but associations with “risk factors” such as RF, anti-CCP and HLA-DR4 status were not found. The MMP-1 and MMP-3 promoter polymorphisms were not associated with radiological damage or radiological progression after a follow-up of 2 years. Serum MMP-3\(^{AUC}\) correlated significantly with radiological progression after 6 (r = 0.26, p < 0.01) and 12 months (r = 0.36, p < 0.01) The sMMP-3\(^{AUC}\) after 6 and 12 months did not differ across the three MMP-3 promoter polymorphism groups. sMMP-3\(^{AUC}\)/CRP\(^{AUC}\) ratios differed after 12 months across the three promoter polymorphism groups with an increase from the 5A/5A to the 6A/6A carrier genotype (p < 0.01).

Conclusion. In our study in early RA patients we could not find an association between MMP-1 and MMP-3 promoter polymorphism and disease activity or severity at presentation. In addition the MMP-1 and MMP-3 promoter polymorphisms were not associated with radiological damage or radiological progression after a follow-up of 2 years.

Key words: matrix metalloproteinase 1, matrix metalloproteinase 3, promoter polymorphism, early rheumatoid arthritis, disease activity, radiological progression.
INTRODUCTION
Rheumatoid arthritis (RA) is a systemic inflammatory disorder characterized by chronic inflammation of synovial tissue and in most cases progressive destruction of cartilage and bone. It affects about 0.5 to 1% of the population and has substantial impact in terms of disability, morbidity, mortality, and costs. Early identification of patients with aggressive destructive disease is important not only for prognostic but also for therapeutic reasons. Novel, more aggressive therapies are currently developed and the need for clinically feasible means for assessing the prognosis in the individual patient is becoming increasingly important. Disease activity, rheumatoid factor positivity, and early radiological abnormalities are considered to be important in relation to outcome such as radiological damage. Genetic studies show associations between radiological damage and HLA-DRB1 shared epitope and recently with matrix metalloproteinase (MMP) promoter polymorphisms.

MMPs are proteolytic enzymes involved in many physiological processes and pathological conditions. These enzymes are capable of degrading many components of the extra-cellular matrix and/or activate other MMPs. It is conceivable that an imbalance between production, activation, and inhibition of MMPs results in matrix degradation.

In RA, the MMPs are locally produced and activated within the affected joint as a result of cytokine mediated stimulation of synovial cells. In particular matrix metalloproteinase 3 (MMP-3) is of interest because this proteolytic enzyme is produced abundantly in the inflamed joints and plays a prominent role in the pathogenesis of matrix degradation in RA, even though it is not the only key-enzyme. MMP-3 is capable of degrading many components of the matrix in the synovial joint including proteoglycans, gelatins, laminin, fibronectin, and collagen III, IV, IX. Moreover, MMP-3 is able to activate other matrix metalloproteinases such as MMP-1, MMP-7, MMP-8, MMP-9 and MMP-13. The enzyme has been localized in the fibroblast-like synoviocytes of rheumatoid synovium, in RA cartilage, at sites of cartilage erosion, in synovial fluid and in serum. Systemic MMP-3 levels are supposed to reflect local synthesis induced by pro-inflammatory cytokines. As such, serum MMP-3 can be used as a systemic marker of local joint inflammation and/or destruction.

Several regulatory mechanisms influence the ultimate effect of a MMP on extra-cellular matrix, such as regulation of transcription, activation of latent proMMPs, and inhibition of MMP activity by inhibitors such as TIMPs (tissue inhibitors of matrix metalloproteinases). At the level of transcription,
functional promoter polymorphisms in the genes of important MMPs might have implications for susceptibility and/or severity of diseases.\textsuperscript{42}

With respect to RA, functional MMP promoter polymorphisms have been reported, which seem to be of importance in the regulation of MMP gene expression.\textsuperscript{42} For example in the promoter region of the MMP-1 gene, at position -1607, two alleles have been detected; one is having a single guanine (1G) and the other having two guanines (2G). In in vitro experiments the 2G allele had substantially more transcriptional activity compared to the 1G allele.\textsuperscript{43,44}

In the promoter of MMP-3 a polymorphism at position -1171 appears to be functionally important, at least in in vitro experiments. Cultured cells transfected with constructs containing a run of 6 adenines (6A) expressed a roughly 2-fold lower amount of reporter gene product as compared with the transfectants of the constructs containing a run of 5 adenines (5A).\textsuperscript{45}

In previous studies in RA patients an association between the 1G-1607/2G promoter polymorphism in the MMP-1 gene and radiological damage could not be confirmed.\textsuperscript{46} However in another study by the same group of investigators an association was found between the 5A-1171/6A promoter polymorphism in the MMP-3 gene and radiological damage and progression.\textsuperscript{8} In particular patients homozygous for the 6A allele had the highest radiological progression. This seems to contrast with in vitro experiments in which this 6A/6A polymorphism expressed a lower amount of gene product.\textsuperscript{42-45}

In the present prospective follow-up study of early RA patients we evaluated the significance of MMP-1 and MMP-3 promoter polymorphisms in relation to disease activity and radiological damage. In a subgroup of patients the association between the MMP-3 promoter polymorphism and serum levels of MMP-3 (sMMP-3) was evaluated.

**PATIENTS AND METHODS**

*Patients*

Patients with early RA who participated in a prospective follow-up study between January 1985 and December 2000 at the Department of Rheumatology of the Groningen University Hospital were included in this study. All patients met the following criteria: a. the 1987 American College of Rheumatology criteria\textsuperscript{47} b. joint symptoms existing less than one year at presentation c. no previous disease modifying anti rheumatic drugs (DMARDs) and d. two years of clinical and radiological follow-up.

Clinical and laboratory investigations were performed at monthly intervals and
radiographs of hands and feet were obtained every 6 months during follow-up.

_Treatment_
During follow-up, patients were treated with non-steroidal anti-inflammatory drugs as indicated clinically. Low dose corticosteroids could be administered as adjuvant therapy. From January 1985 until January 1991 the guidelines for the sequence of the different DMARDs were as follows: Hydroxychloroquine or Sulphasalazine (SASP) as first choice therapy, followed in order by intramuscular Gold, D-penicillamine, Azathioprine, or Methotrexate (MTX). These patients are denoted as traditional treatment cohort. From January 1991 until December 1993, patients participated in a study in which this traditional regime was compared with an early, intensive drug treatment. Since January 1994 it is our policy to use this intensive treatment strategy in early RA patients consisting of an immediate start of DMARDs and rapid adjustment of dosage and/or drugs (step up method) in case of an insufficient response. In this intensive treatment cohort all patients started with SASP 2000-3000 mg/day. In case of an insufficient response MTX, in increasing dosages up to 25 mg/week, could be added. If there was still an insufficient response, SASP was replaced by either cyclosporine or more recently by a TNF-α blocking agent.

Decisions about intensifying the treatment with DMARDs were discussed at monthly visits by an independent observer and the patient’s rheumatologist. These decisions were based on clinical markers of disease activity in combination with the CRP level as effect measures. A sufficient response was defined as a ≥ 50% reduction in joint scores or CRP level (or normalization of CRP (<2 mg/l)).

_Clinical markers of disease activity_
Fifty-two peripheral joints were examined for tenderness and soft tissue swelling. The following articular indices were determined: Ritchie articular index (RAI), tender joint count (TJC), swollen joint count (SJC), and the disease activity score (DAS) according to Van der Heijde with 3 variables: RAI, number of swollen joints, and erythrocyte sedimentation rate (ESR). The maximal scores are: RAI 78, TJC 52, SJC 52 and DAS 10.0.

_HLA-DR4 typing_
HLA typing was performed by serologically using two coloured fluorescence technique, and since 1994 by DNA using sequence specific primers. In case only a single HLA-DR4 allele was found, the alleles were assumed to be present
homozygously.

**MMP-1 and MMP-3 polymorphism genotyping**

Genomic DNA from each individual was isolated from a whole blood sample by using the Puregene® DNA isolation kit (Gentra Systems, Minneapolis, USA). DNA from smaller amounts of frozen lymphocyte suspensions was isolated using the QIAmp DNA Blood mini kit (QIAGEN, Hilden, Germany).

The final DNA samples were measured by using a Bio Photometer (Eppendorf, Hamburg, Germany) calculating the concentration and purity of the samples. For MMP polymorphism analyses the Taqman assay (Applied Biosystems, Foster City, USA) was used. Briefly this assay enables the discrimination of two alleles based on the hybridization of labeled specific probes and the 5’-3’ exonuclease activity of Taq DNA polymerase. The exonuclease will digest hybridized probes, separating the reporter and quencher, leading to one or two specific fluorescence signals. Primer and labeled probes for MMP-1 polymorphism (at position -1607) and MMP-3 polymorphism (at position -1171) were developed by Applied Biosystems (Assay by Design). The sequences for MMP-1 primers were 5’-GTT ATG CCA CTT AGA TGA GGA AAT TG (forward) and 5’-CAT AAA CAA TAC TTC AGT ATA TCT TGG ATT GA (reverse), and for MMP-3 the primers were 5’-TGG TTC TCC ATT CCT TTG ATG GG (forward) and 5’-TCA ATG TGG CCA AAT ATT TTC CCT GTA (reverse). The dye-labeled probes for MMP-1 were 6-FAM-AGT CAT ATC TTT CTA ATT AT-NFQ-MGB (1G) and VIC-AGA TAA GTC ATA TCC TTT CT-NFQ-MGB (2G) in which NFQ is the Non-Fluorescent Quencher, FAM and VIC the reporter dye, and MGB the Minor Groove Binder for increase of annealing temperature to enable use of shorter probes. The FAM probe detects the 1G and the VIC probe the 2G polymorphism of MMP-1. For MMP-3 the 5A allele was detected by the 6-FAM-AAG ACA TGG TTT TT C-NFQ-MGB probe and the 6A allele by the VIC-AAG-ACA-TGG- TTT-TTT-C-NFQ-MGB probe.

Genomic DNA (10 ng) was added to a 384-well microtitre plate and dried. The polymerase chain reaction (PCR) and measuring of the fluorescence was performed under standard conditions after adding 5 µl reaction mix (containing Taqman Universal Master Mix, primers and probes) by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA). The intensities of the allele specific fluorophores were plotted against each other and 3 groups were identified as shown in figure 1A & B.
FIGURE 1. Representation of the results of the genotyping for (A) MMP-1 and (B) MMP-3. In both plots three groups were identified. H2O controls are water controls.

*Serum MMP-3*

Serum MMP-3 (sMMP-3) levels were determined by a MMP-3 ELISA developed at our laboratory. In short, 96 well plates were precoated with F(ab)2 fragment of goat-antimouse IgG, 1µg/ml (Jackson Immunoresearch Labs, West Grove, PN, USA). Next a mouse monoclonal antibody against human MMP-3, clone 10D6 (R&D Systems, Abingdon, UK) was coated at 0.1 µg/ml. Serum samples were analyzed in two-fold serial dilutions in high performance ELISA buffer (CLB, Amsterdam, NL) and incubated during 1 hour. After washing bound MMP-3 was detected with a polyclonal rabbit-anti-human MMP-3 (AB 810, Chemicon, Temecula, CA, USA), followed by horseradish-peroxidase-labelled F(ab)2 fragment of goat-anti-rabbit IgG (Zymed, San Francisco, CA, USA). Peroxidase activity was determined using tetramethylbenzidin as substrate. MMP-3 levels were calculated at the linear range of the assay from a standard curve (3-400 ng/ml) using pro-MMP-3, purified from serum free supernatant of IL-1β stimulated rheumatoid arthritis synovial fibroblasts. The intra-assay coefficient of variation (CV) was 6.8%, the inter-assay CV 8.8%. With an immunoblot we demonstrated that both the monoclonal and the polyclonal antibody reacted with active MMP-3, pro-MMP-3 as well as with MMP-3 bound to tissue inhibitor of matrix metalloproteinases (TIMPs) (data not shown). Furthermore it was demonstrated that rheumatoid factors do not react in this assay and do not interfere with measurement of MMP-3 (data not shown).
For normal values of sMMP-3 the 95 percentile in healthy bloodbank donors (n=80) was used (female < 20 ng/ml, male < 60 ng/ml).

**Anti-CCP**

Serum anti cyclic citrullinated peptides (anti-CCP) levels were determined using an ELISA kit as described by the manufacturer (Euro-Diagnostica, Arnhem, NL). Serum or plasma was added to microtitre plate wells coated with citrullinated synthetic peptides. The bound antibodies are detected by adding a HRP labelled antibody to human IgG and subsequently visualized with a color reaction. The measured absorbency of the unknown samples is calculated against a standard curve, ranging from 25-1600 Units/ml., using the software package Softmax Pro (Molecular Devices, Sunnyvale, USA). Normal or negative values are defined as ≤ 25 Units/ml.

**CRP, ESR and Rheumatoid factor**

C-reactive protein (CRP) was measured by ELISA (normal value: < 2 mg/l), ESR according to Westgren. IgM rheumatoid factor (RF) was measured by Dade/Behrng BN-2 nephelometer (normal value: < 15 IU/ml).

**Radiological analysis**

Radiological damage in hands and feet was assessed by Sharp’s method with some modifications as described by Van der Heijde et al. By this method joint space narrowing (JSN) and erosions (ER) are scored separately and combined to a total Sharp score (TSS) with a maximum TSS of 448 points. The radiographs were scored without knowledge of clinical and laboratory data in chronological order per patient by two observers. In a random sample of 20 patients the inter-observer agreement was 0.90 and the intra-observer agreements were 0.96 and 0.99 for the two observers respectively.

**Statistical analysis**

To investigate the association between MMP promoter polymorphisms and quantitative markers of disease activity, such as the DAS and CRP, and radiological damage (TSS) at presentation the Kruskal-Wallis test was used. For evaluation of an association between MMP promoter polymorphisms and other “riskfactors” at presentation such as HLA-DR4, RF and anti-CCP the Chi-square test was applied. Linkage disequilibrium between MMP-1 and MMP-3 promoter polymorphism was evaluated using the software package Arlequin. To determine the additional value of MMP promoter polymorphisms to the
MMP-1 and MMP-3 promoter polymorphism in early RA

prediction of progression of radiological damage (Δ TSS) an univariate generalized linear model was used with incorporation of all other relevant prognostic factors.

To investigate the significance of MMP-3 promoter polymorphism with respect to sMMP-3 levels monthly sMMP-3 (and CRP) levels were plotted against time and time-integrated values were obtained by calculation of the area under the curve. Cumulative values were determined over 6 months periods (sMMP-3^{AUC} and CRP^{AUC}). The association between MMP-3 promoter polymorphism and sMMP-3^{AUC} and MMP-3^{AUC}/CRP^{AUC} ratios during respectively the first 6 and 12 months of follow-up was evaluated by using the Kruskal-Wallis test.

To evaluate the relation between sMMP-3^{AUC} over 6 months periods and radiological progression during the first year of follow-up the Spearman’s rank correlation coefficient was determined.

Statistical analysis was carried out using the SPSS statistical package, 10.0.

RESULTS

Table 1 shows the characteristics of the 448 early RA patients at presentation.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the study population at presentation (n = 448).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> yrs.</td>
</tr>
<tr>
<td>53.3</td>
</tr>
<tr>
<td><strong>Anti-CCP</strong> % +</td>
</tr>
<tr>
<td>75.4 %</td>
</tr>
<tr>
<td><strong>MMP-3 polymorphism</strong> 5A/5A 5A/6A 6A/6A</td>
</tr>
<tr>
<td>25.0 % 52.8 % 22.2 %</td>
</tr>
<tr>
<td><strong>SJC</strong> 11</td>
</tr>
<tr>
<td>0-36</td>
</tr>
<tr>
<td><strong>DAS</strong> 6.4</td>
</tr>
<tr>
<td>3.2 - 10.0</td>
</tr>
<tr>
<td><strong>ESR</strong> mm/hr 36</td>
</tr>
<tr>
<td>2 - 130</td>
</tr>
</tbody>
</table>

Demographic and clinical data of the patients at study entry. The absolute values are the median and range. SJC; swollen joint count. TJC; tender joint count. RAI; Ritchie articular index. DAS; disease activity score with 3 variables. % +; percentage positive. % 0; percentage with Sharp score of 0.
Suitable materials to determine MMP-1 and MMP-3 promoter polymorphisms were available in 394 and 396 patients respectively. The described assay to determine MMP-1 and MMP-3 promoter polymorphism identified 3 groups (figure 1) with frequencies (see table 1) comparable to the literature\textsuperscript{8,46}.

In table 2 the MMP-1 and MMP-3 promoter polymorphisms are described in detail. Haplotypes were not determined and thereby unknown in 120 patients. However by using the Arlequin software package strong linkage disequilibrium between the two promoter polymorphisms of MMP-1 and MMP-3 was found (p < 0.001). The 1G and 2G alleles were more frequently (than expected) linked to the 5A and 6A alleles respectively.

There were no associations between MMP-1 and/or MMP-3 promoter polymorphism and RF, anti-CCP, and HLA-DR4 status at presentation (Chi square).

<table>
<thead>
<tr>
<th>TABLE 2. MMP-1 and MMP-3 promoter polymorphisms in the study population (n=448).</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1G/1G</td>
</tr>
<tr>
<td>5A/5A</td>
</tr>
<tr>
<td>5A/6A</td>
</tr>
<tr>
<td>6A/6A</td>
</tr>
<tr>
<td>nd #</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*# nd; not done = inadequate material to determine promoter polymorphisms.*

*MMP-1 and MMP-3 promoter polymorphism in relation to activity and severity of RA at presentation*

The DAS at presentation did not differ among the three MMP-1 and the three MMP-3 polymorphism groups. Analysis of the DAS at presentation in subgroups stratified by RF, anti-CCP status, HLA-DR4 positivity, or a total Sharp score of \(\leq\) or \(>\) 10 points at presentation did not show any significant differences either.

The same analysis was performed with the CRP level, total Sharp score and serum MMP-3 level at presentation. The distribution of all these variables did not significantly differ across the different MMP-1 or MMP-3 promoter polymorphism groups, neither in the whole group nor in subgroups.

A subanalysis of the two items of the total Sharp score, erosions and joint space narrowing did not reveal significant differences across the MMP-1 or MMP-3 genotypes (table 3).
MMP-1 and MMP-3 promoter polymorphism in relation to radiological progression

In an univariate generalized linear model no association was found between the MMP-1 or MMP-3 promoter polymorphisms and radiological progression (Δ TSS) or the TSS at 24 months. In contrast, variables such as treatment strategy, anti-CCP status, ESR, CRP and TSS at presentation were significantly associated with ΔTSS and the absolute TSS at 24 months (data not shown).

Because of clear differences in TSS and ΔTSS (table 4) a subgroup analysis was done among the different treatment strategy groups. However, this did not reveal significant associations between MMP-1 or MMP-3 promoter polymorphisms and ΔTSS or the absolute TSS at 24 months.

**TABLE 3.** MMP1 and MMP-3 promoter polymorphisms and radiological severity at presentation.

<table>
<thead>
<tr>
<th></th>
<th>MMP-1</th>
<th></th>
<th>MMP-3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1G/1G</td>
<td>1G/2G</td>
<td>2G/2G</td>
<td>5A/5A</td>
</tr>
<tr>
<td>Baseline</td>
<td>(n=114)</td>
<td>(n=201)</td>
<td>(n=79)</td>
<td>(n=99)</td>
</tr>
<tr>
<td>TSS 1G/1G</td>
<td>1 (0-3)</td>
<td>1 (0-4)</td>
<td>1 (0-6)</td>
<td>1 (0-5)</td>
</tr>
<tr>
<td>ER 1G/1G</td>
<td>0 (0-2)</td>
<td>1 (0-3)</td>
<td>1 (0-3.5)</td>
<td>0 (0-3)</td>
</tr>
<tr>
<td>JSN 1G/1G</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td></td>
<td>(n=14)</td>
<td>(n=15)</td>
<td>(n=7)</td>
<td>(n=9)</td>
</tr>
</tbody>
</table>
| Median and interquartile range of the Total Sharp Score (TSS), Erosions (ER) and Joint Space Narrowing (JSN) at presentation in the MMP-1 and MMP-3 promoter polymorphism groups.

These variables were not different across the MMP-1 and MMP-3 polymorphism groups. (Kruskal-Wallis test).

**MMP-3 promoter polymorphism in relation to sMMP-3**

Monthly sMMP-3 levels in combination with MMP-3 promoter polymorphism were available in 99 patients during the first 6 months and in 66 patients during 12 months. To investigate the significance of an MMP-3 promoter polymorphism with respect to serum levels monthly sMMP-3 levels were plotted against time and time-integrated values were obtained by calculation of the area under the curve (sMMP-3\(^{AUC}\)). Cumulative values were determined over 6 months periods.

This sMMP-3\(^{AUC}\) after 6 and 12 months correlated significantly with ΔTSS

**TABLE 4.** Total Sharp score and radiological progression in different treatment groups.

<table>
<thead>
<tr>
<th></th>
<th>TSS</th>
<th>Δ TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Traditional treatment</td>
<td>1</td>
<td>0-4.5</td>
</tr>
<tr>
<td>Intermediate group</td>
<td>2</td>
<td>0-6</td>
</tr>
<tr>
<td>Intensive treatment</td>
<td>1</td>
<td>0-3</td>
</tr>
<tr>
<td>All patients</td>
<td>1</td>
<td>0-4</td>
</tr>
</tbody>
</table>

Total sharp score (TSS) at presentation (0 months), after 12 and 24 months of follow-up and radiological progression after 24 months (Δ TSS) in the 3 different treatment groups.

¶ P < 0.001 compared to traditional and intermediate treatment group (Kruskal-Wallis test).
after 6 months (Spearman’s $r = 0.26$, $p < 0.01$) and after 12 months ($r = 0.36$, $p < 0.01$).

The sMMP-3$^{\text{AUC}}$ after 6 and 12 months did not differ across the MMP-3 promoter polymorphism groups (Kruskal-Wallis test). There was a trend to a higher sMMP-3$^{\text{AUC}}$ in the 6A/6A group but this did not reach statistical significance (figure 2).

**FIGURE 2.** Serum MMP-3 and CRP area under the curve (AUC) after 6 and 12 months of follow-up across the MMP-3 promoter polymorphisms. Statistical significant differences were not found (Kruskal-Wallis) although the sMMP-3$^{\text{AUC}}$ had a tendency to higher levels in the 6A/6A group.
MMP-3 promoter polymorphism in relation to sMMP-3\(^{AUC}/\text{CRP}^{AUC}\) ratios

Serum MMP-3 and CRP are closely correlated in patients with RA \(^{28,33,37,59}\). To evaluate an additive effect of MMP-3 promoter polymorphism “correction for inflammation” was made by evaluation of sMMP-3\(^{AUC}/\text{CRP}^{AUC}\) ratios. After 6 months of follow-up the ratios were not different across the MMP-3 promoter polymorphism groups. However after 12 months a significant difference in ratios was found (p < 0.01) between the 5A/5A & 5A/6A and 5A/5A & 6A/6A groups (Kruskal-Wallis with Dunn’s multiple comparison test) (figure 3).

**FIGURE 3.** Serum MMP-3 area under the curve/CRP area under the curve (AUC) ratio’s across the MMP-3 promoter polymorphism groups after 6 and 12 months of follow-up. After 6 months of follow-up the ratios were not different across the MMP-3 promoter polymorphism groups. After 12 months a significant difference in ratio’s was found (p < 0.01) determined by a difference between the 5A/5A & 5A/6A (p < 0.01) and 5A/5A & 6A/6A groups (p < 0.01) (Kruskal-Wallis with Dunn’s multiple comparison test).

**DISCUSSION**

In the present study concerning 448 early RA patients with a follow-up of 2 years we could not find any association between MMP-1 and MMP-3 promoter polymorphism and disease activity or severity at presentation. MMP-1 and MMP-3 promoter polymorphisms showed strong linkage disequilibrium, but associations with “risk factors” such as RF, anti-CCP, and HLA-DR4 status were not found. In addition the MMP-1 and MMP-3 promoter polymorphisms were not associated with radiological damage or radiological progression after a follow-up of 2 years. Because aggressive treatment seems to influence the
association between genetic markers and radiological damage \(^60\) a subgroup analysis was done in the 3 different treatment groups with clearly different radiological progression (table 4). This analysis did not reveal different results.

In accordance with previous studies we confirmed that the cumulative serum MMP-3 designated by the serum MMP-3 area under the curve (sMMP-3\(^{\text{AUC}}\)) is correlated with radiological progression \(^37\). The sMMP-3\(^{\text{AUC}}\) after 6 and 12 months of follow-up was however not different across the three MMP-3 promoter polymorphism groups. sMMP-3\(^{\text{AUC}}/\text{CRP}^{\text{AUC}}\) ratios differed after 12 months across the three promoter polymorphism groups with an increase from the 5A/5A to the 6A/6A carrier genotype possibly due to the higher (although not statistically significant) sMMP-3\(^{\text{AUC}}\) in this group.

In RA the MMPs are important mediators in the process of joint destruction. \(^61\) In particular MMP-3 is of interest because of its key role in the destructive process \(^10,11,13\) and its associations with disease activity \(^32,33,36,37\) and joint damage \(^34,38,39\). Most MMPs are inducible and their production can be regulated at different levels such as signal transduction preceding transcription, transcription itself, or posttranscriptional processing. At the level of transcription functional promoter polymorphisms in the genes of important MMPs might have implications for disease susceptibility and/or severity of various diseases \(^42\).

From \textit{in vitro} experiments it appears that a polymorphism in the promoter region of the MMP-1 may be of functional importance. In the MMP-1 gene (locus 11q22-q23) two alleles have been detected; one having a single guanine (1G) and the other having two guanines (2G) at position -1607 (1G-1607/2G). The 2 guanines together with an adjacent adenine can create a core binding site (5'-GGA-3') for the Ets family of transcription factors. This 2G allele binds substantially more Ets-1 and has substantially more transcriptional activity compared to the 1G allele in normal fibroblasts and melanoma cells \(^43\) as well as in ovarian tumor tissue \(^44\). Influences of this promoter polymorphism have been evaluated in cancer \(^42,62\), pulmonary disease \(^63\), and scleroderma \(^64\). In RA patients Constantin et al could not find an association between this polymorphism in the MMP-1 promoter and the susceptibility to or severity of RA \(^46\).

With respect to our data in early RA patients the results concerning the MMP-1 promoter polymorphism are consistent with previous reported studies in pulmonary disease \(^63\), scleroderma \(^64\), and RA \(^46\). These studies and our results do not support the hypothesis of an association between this MMP-1 promoter polymorphism and disease activity and severity of RA.

Another, \textit{in vitro} functional, polymorphism has been described in the promoter region of the MMP-3 gene (locus 11q23), at position -1171 (5A-1171/6A).
Cultured fibroblasts and vascular smooth muscle cells transfected with constructs containing the run of 6 adenines (6A) expressed a roughly 2-fold lower amount of reporter gene product as compared with the transfectants of the constructs containing a run of 5 adenines (5A) \(^{45}\). Additional studies revealed binding of putative transcription factors to this region. The difference in promoter activity is probably due to preferential binding of a transcription repressor to the 6A allele \(^{45,65}\). Influences of this polymorphism have been evaluated in atherosclerosis \(^{66-69}\) and cancer \(^{70,71}\). In RA patients, Constantin et al found no association between the 5A-1171/6A polymorphism and disease susceptibility. However severity and progression of RA, represented by radiologically detectable joint damage, appeared to be associated with the 6A/6A genotype \(^8\), which in \textit{in vitro} experiments appears to be associated with a lower transcriptional activity, compared to the 5A carrier genotype.

Our data concerning the MMP-3 promoter polymorphism differ from the study by Constantin et al \(^8\). In contrast to our results they found a significant association between MMP-3 promoter polymorphism and radiological damage after 4 years of follow-up of 96 RA patients. This discrepancy could be due to a number of reasons.

Firstly, there is a difference in duration of follow-up between the two studies, with a follow-up of 2 years in our study and a follow-up of 4 years in the study of Constantin. Theoretically a relative small effect on radiological damage due to e.g. an MMP-3 promoter polymorphism can eventually become statistically significant if the follow-up is long enough. On the other hand such a statistical significance after a long follow-up would imply a decreased clinical relevance.

Secondly, differences in disease activity could be of importance. For example the mean disease activity score in the study of Constantin et al at presentation was 2.8 (SD ± 1.1) and in our study 6.4 (SD ± 1.4). Many of their patients (41\%) already used DMARDs in contrast with our patients who were DMARD-naive. High disease activity with active cytokine networks and signal transduction pathways causing extensive mRNA expression of multiple MMP genes could theoretically overrule a relatively small difference in production due to promoter polymorphisms. This last mechanism might also explain why we could not find a significant difference in sMMP-3\(^{AUC}\) across the different MMP-3 promoter polymorphism groups.

Thirdly, it is striking that, in the study of Constantin et al, there seems to be an association between the \textit{in vitro} “low producer” 6A/6A promoter polymorphism and radiological damage. In our study the sMMP-3\(^{AUC}\) was not statistically different across the polymorphism groups although the levels tended to increase.
towards the 6A/6A genotype (figure 2). The \( \text{sMMP-3}^{\text{AUC}}/\text{CRP}^{\text{AUC}} \) ratios, used as a “correction for inflammation” were significantly higher in the 5A/6A and 6A/6A groups after 12 months. So *in vivo*, in early RA the 5A/6A and 6A/6A genotypes appear to produce more MMP-3 relative to CRP. A straightforward explanation for these conflicting data is not available but apparently important differences exist between *in vitro* experiments and *in vivo* disease with regard to regulatory mechanisms of transcription as well as pre- and posttranscriptional processes.

Finally, linkage with other MMPs could be of influence. At chromosome 11q21-23 at least 8 known human MMP genes (MMP-1, -3, -7, -8, -10, -12, -13 and -20) are clustered and, for example the MMP-1 locus is adjacent to the MMP-3 locus. MMP-1 and MMP-3 promoter linkage disequilibrium has been reported. In colorectal cancer the 2G MMP-1/6A MMP-3 haplotype was significantly increased\(^7\) and in RA patients Keyszer et al reported that almost all haplotypes were found to be either 1G MMP-1/5A MMP-3 or 2G MMP-1/6A MMP-3\(^9\). These last data are in line with our own results with a more frequent prevalence of the 1G/5A and 2G/6A combinations. So linkage in this region is demonstrated and possibly of importance. Theoretically the 5A-1171/6A MMP-3 promoter polymorphism could be linked to other much more important MMP genes with respect to the destructive processes in RA. In that case the described association between the 5A-1171/6A MMP-3 promoter polymorphism and radiological damage might be just an epiphenomenon.

Despite the fact that were not able to demonstrate any association between MMP-1 or MMP-3 promoter polymorphism and radiological progression, \( \text{sMMP-3}^{\text{AUC}} \) itself appeared to be correlated with radiological progression after 6 and 12 months. This confirms previous results from our\(^37\) and other groups\(^38,39\) that serum MMP-3 is associated with radiological progression in early RA. These results underline serum MMP-3 as an intriguing marker of disease severity.

In conclusion in our study we could not find an association between MMP-1 and MMP-3 polymorphism and disease activity or severity at presentation. In addition the MMP-1 and MMP-3 promoter polymorphisms were not associated with radiological damage or radiological progression after a follow-up of 2 years. However MMP-3 promoter polymorphisms 5A/6A and 6A/6A were associated with increased serum MMP-3 levels relative to CRP, as analyzed over a 12 months period. Although cumulative MMP-3 levels are associated with radiological progression in RA, the influence of the MMP-3 promoter polymorphism is apparently not of clinical relevance.
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