Macrophage migration inhibitory factor (MIF) and oligoarticular juvenile idiopathic arthritis (o-JIA): association of MIF promoter polymorphisms with response to intra-articular glucocorticoids

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Abstract

Objectives

To address the clinical relevance of macrophage migration inhibitory factor (MIF) promoter polymorphisms in oligoarticular juvenile idiopathic arthritis (o-JIA) by evaluating their associations with serum and SF MIF levels, with response to intra-articular glucocorticoid injections and with outcome of the disease.

Methods

Seventy-five Caucasian patients with o-JIA were studied. Alleles of the -794 CATT variable number of tandem repeats (VNTR) and of the -173 G/C single nucleotide polymorphism (SNP) were identified by capillary electrophoresis following fluorescently labelled PCR and by allelic discrimination assay, respectively. MIF levels were measured by ELISA. The association of MIF promoter polymorphisms with polyarticular extension, Childhood Health Assessment Questionnaire (CHAQ) score at the last follow-up visit and occurrence of chronic anterior uveitis was evaluated only in patients with a follow up > 5 years.

Results

Neither of the MIF promoter polymorphisms was associated with serum MIF levels, nor with the long-term outcome of o-JIA. The -173 G/C SNP was significantly associated with both SF MIF levels and duration of response to intra-articular glucocorticoid injection. Carriers of a MIF -173*G allele were 4 times more likely to relapse within 3 months. No association was found between the different MIF CATT alleles and both SF MIF levels and duration of response to intra-articular glucocorticoids.

Conclusions

Our study shows the clinical relevance of the MIF -173 G/C SNP in o-JIA and suggests that the -173*G allele may represent a predictor of poor response to intra-articular glucocorticoid treatment.

Key words

MIF, polymorphism, arthritis, glucocorticoid.
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**Introduction**

Oligoarticular juvenile idiopathic arthritis (o-JIA) is the most common form of JIA, accounting for 30 to 60% of the whole patient population (1, 2). It is defined by the presence of arthritis in 4 or less joints during the first 6 months of disease (3). Extension of articular involvement to 5 or more joints (polyarticular course) is an important determinant of long-term outcome (4). The articular inflammatory process of o-JIA has been associated with elevated synovial levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (5). Standard treatment for o-JIA includes intra-articular glucocorticoid injections with triamcinolone hexacetonide (TXA), which has been shown to be safe and rapidly effective; however, patient response to this treatment varies widely (6).

MIF is a proinflammatory cytokine that plays a key role both in innate and in adaptive immunity (7) and has been implicated in a variety of diseases involving immune dysregulation (8, 9). MIF exerts several pro-inflammatory effects, including upregulation of TLR4 expression, activation of ERK phosphorylation, which induces arachidonic acid release, and interaction with the intracellular protein JAB1, which leads to inhibition of apoptosis (10, 11). In addition, MIF has the unique ability to override the inhibitory effects of glucocorticoids on the immune system. This has been proven both in *vitro*, on proinflammatory cytokine secretion by LPS-stimulated monocytes (12) and on T cell proliferation and production of IL-2 and IFN-γ (13), and in *vivo*, in models such as lethal endotoxemia (14) and antigen-induced arthritis (15). These observations suggest that MIF production represents a physiologic counter-regulator of glucocorticoid inhibitory effects on immune responses. The mechanism underlying this effect of MIF was recently shown to involve the phosphatase MKP-1, a key element in inactivating transcription of several pro-inflammatory mediators whose expression is increased by glucocorticoids (16).

MIF appears to play a pivotal pro-inflammatory role within the joint: a) it is expressed in synovial tissue of patients with RA and its expression is down-regulated by glucocorticoids (17), b) *in vitro* it induces transcription of numerous inflammatory mediators by synovial fibroblasts of patients with RA (18-20), c) in animal models of arthritis MIF neutralization results in a drastic reduction of inflammation (21-23) and of leukocyte recruitment to the synovium (24). MIF levels are elevated also in patients with all forms of JIA (25). These results suggest an involvement of MIF in the pathogenesis of different forms of arthritis.

Two polymorphisms have been identified in the promoter region of the MIF gene (26): the variable number (5 to 8) of CATT repeats (CATT<sub>5-8</sub>) at position -794 and the single nucleotide G to C polymorphism at position -173. In adults, the MIF -173*C and the CATT<sub>5-8</sub> alleles have been associated with increased circulating MIF levels and correlated with greater radiologic joint damage in RA (27). In children, the -173*C allele has been shown to confer increased susceptibility to all forms of JIA (26). This polymorphism is functionally relevant: a) *in vitro* the -173*C allele increases MIF expression in a lymphoblast cell line (26), b) *in vivo* the -173*C allele is associated with higher serum MIF levels both in controls and in patients with systemic JIA (26, 28). Moreover, in systemic JIA the presence of the -173*C allele appears to correlate both with disease severity and with decreased response to glucocorticoid treatment (28).

Increased serum and SF levels of MIF have been found in o-JIA (25). In this study, we investigated the clinical relevance of MIF promoter polymorphisms in o-JIA by evaluating their association with serum and synovial levels of this cytokine, with response to intra-articular glucocorticoids and with long-term disease outcome.

**Materials and methods**

**Study design, patients and treatment**

This was a retrospective cohort study in which we included a total of 75 patients with o-JIA, diagnosed according to the International League of Associations for Rheumatology (ILAR) criteria (3), whose genomic DNA was available.
Patients were followed in part first at the IRCCS Policlinico San Matteo, Pavia, and subsequently at the IRCCS G. Gaslini, Genova, and in part at the IRCCS Ospedale Pediatrico Bambino Gesù, Rome.

No patient included in the study was receiving oral glucocorticoids at the time of serum or synovial fluid (SF) sampling. Clinical records were examined by observers (M.V. and A.I.) who were blinded to the MIF genotype of patients. The association of MIF promoter polymorphism with clinical outcome was evaluated only in patients with disease duration greater than 5 years. For this purpose, blinded observers (M.V. and A.I.) retrospectively reviewed patients' clinical charts to record the following information: a) polyarticular extension (i.e. involvement of 5 or more joints); b) level of functional ability at last follow-up visit, measured by the Childhood Health Assessment Questionnaire (CHAQ) (29); c) chronic anterior uveitis. This information was reported on a standard form.

Intra-articular triamcinolone hexacetonide (TXA) was administered at previously described doses (28). Joints injected with other glucocorticoids were excluded from the study. A total of 286 intra-articular administrations of TXA performed in 60 patients with o-JIA were assessed. Blinded observers (M.V. and A.I.) calculated the number of months of complete clinical response defined as absence of active arthritis (30) after each joint injection, as recorded in our standardized joint assessment form, completed at each clinical visit.

Given the recent demonstration of ethnic variation in MIF allele frequencies in different populations (31), it is important to note that all patients and controls were Caucasians of Italian descent. All procedures involving patients and controls were in accordance with the standards of the responsible local ethics committee.

Genotyping for the MIF promoter polymorphisms
Genomic DNA was extracted from all samples using minirePeACE MaxiBlood Purification System kits (Bioline, London, UK).

**MIF CATT repeats**: Alleles of the CATT repeat element were identified using a fluorescently labelled PCR primer and capillary electrophoresis. Genomic DNA (20ng) was amplified by PCR in a total reaction volume of 10ul containing 5pmol of both the forward and reverse primers: (Forward primer 5’ TTG CAC CTA TCA GAG ACC 3’; Reverse primers 5’ TCC ACT AAT GGT AAA CTC G 3’). The forward primer was pre-labelled with a FAM fluorescent dye. Four nmol of each of the four deoxyribonucleotide triphosphates (dNTPs), 0.2 units Taq polymerase (Bioline), 1.5mM MgCl2 buffer 1X KCl buffer and 1mM betaine were included in the PCR mix. The PCRs were performed in 96-well microtitre plates on a Tetrac thermal cycler. Forty PCR cycles were carried out each with denaturation (1 min) 95°C, primer annealing 54°C (1 min) and extension 45 sec at 72°C. A final extension step was conducted at 72°C for 5 mins. Amplified product was pooled with the Tamra 350 size standard (PE Biosystems). Gel electrophoresis was performed on a 0.4mm 6% polyacrylamide gel on a PE Biosystems. Gel was run at 1200V for 2hrs. All results were manually checked using Genotyper 3.6 software.

**MIF -173**: Genotyping was performed using an Assays-on-Demand (SNP ID hCV2213785; AB Biosystems) allelic discrimination assay on a Taqman 7700 platform according to manufacturers' instructions, except that a 10ul rather than a 25ul reaction volume was used (http://www.appliedbiosystems.com). Briefly, the PCR reaction contained 10ng genomic DNA, 2.5ul Taqman master mix and 0.25ul of 40X assay mix. PCR was performed using 384 well plates on an ABI 9700 thermal cycler (reaction conditions 50°C for 2 minutes 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). The Taqman 7700 was used to perform end plate reading using the allelic discrimination option. MIF allele promoter frequencies in the o-JIA patients studied are shown in Table I. In our population, no patients with the MIF -173C/C genotype or with the CATT8 VNTR allele were present. No deviation from Hardy-Weinberg equilibrium was seen for the MIF -173G/C or for the CATT5,8 polymorphisms.

For the -173 locus we analyzed our population by comparing G/G homozygous patients with C allele carriers. Previous studies have shown that the 2-locus haplotype CATT8, MIF-173°C is linked to JIA, suggesting a functional promoter haplotype (32). Therefore, for the -794 CATT5,8 locus, we analyzed our population by comparing CATT8 or CATT5 (CATT5,8) carriers with CATT-carriers. Recently, various studies have shown a correlation between CATT8 and mild asthma (33) and between the CATT8 and a milder clinical expression of cystic fibrosis (34). For this reason, we also performed analysis comparing MIF CATT8 carriers with CATT5,8 carriers.

**Measurement of MIF levels**
Serum and synovial fluid MIF levels were measured with an ELISA as described (25). The detection limit of the assay was 31.25 pg/ml. All the measurements were performed in triplicates.

**Statistical analysis**
Continuous variables were described as median, minimum and maximum value, and compared by non-parametric Mann-Whitney U test. Proportions were compared by Chi-square test or Fisher Exact test, as appropriate. The Chi-square for trend test was used to compare the duration of response in the different MIF genotypes. Helix Tree (Golden Helix, Inc, Bozeman, USA) was used to check Hardy-Weinberg equilibrium.

### Table I. Allele frequencies of the MIF promoter polymorphisms in our population of o-JIA patients.

<table>
<thead>
<tr>
<th>MIF polymorphism allele Patient number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-173G/G                                  53 (70.7%)</td>
</tr>
<tr>
<td>-173G/C                                  22 (29.3%)</td>
</tr>
<tr>
<td>-173C/C                                  0 (0%)</td>
</tr>
<tr>
<td>CATT5/CATT5                              5 (6.7%)</td>
</tr>
<tr>
<td>CATT5/CATT6                              19 (25.3%)</td>
</tr>
<tr>
<td>CATT7/CATT6                              36 (48%)</td>
</tr>
<tr>
<td>CATT7/CATT6                              7 (9.3%)</td>
</tr>
<tr>
<td>CATT7/CATT7                              8 (10.7%)</td>
</tr>
<tr>
<td>CATT7/CATT7                              0 (0%)</td>
</tr>
</tbody>
</table>

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Linear regression was used to evaluate the relation of MIF -173 and -794 CATT\textsubscript{(5,8)} genotypes with duration of the clinical response to intra-articular administration of TXA; a population-averaged model (STATA, Stata Corporation, College Station, Texas, USA) was chosen to obtain weighted estimates adjusting for differences in the number of joints injected in each patient. Since the distribution of durations was right-skewed, the log of duration was used as the analysis variable.

**Results**

**Serum and SF MIF levels and MIF promoter polymorphisms**

Serum MIF levels in subgroups of patients carrying different alleles were compared. When the -173 G/C polymorphism was considered, no difference was found between G/G homozygous patients (median MIF level: 7.71 ng/ml, min-max: 0.6-116 ng/ml) and carriers of a MIF -173*C allele (median MIF level: 9.1 ng/ml, min-max: 0.8-25.8 ng/ml) \( (p = 0.67) \). When the CAAT\textsubscript{(5,8)} repeat was repeated, no difference in serum MIF levels was found between carriers of CATT\textsubscript{(5,6)} (median MIF level: 8 ng/ml, min-max: 0.8-53.8 ng/ml) and carriers of a CATT\textsubscript{(6,7)} allele (median MIF level: 7.75 ng/ml, min-max: 0.6-116 ng/ml) \( (p = 0.6) \). Furthermore, comparison of patients with a CATT\textsubscript{(6)} allele (median MIF level: 8.5 ng/ml, min-max: 0.8-116 ng/ml) allele with patients with a CATT\textsubscript{(6,7)} allele (median MIF level: 6.75 ng/ml, min-max: 0.6-81 ng/ml) showed no difference in serum MIF levels \( (p = 0.54) \). When SF were analysed, carriers of the -173*C allele of MIF showed significantly higher levels of synovial MIF (median: 13 ng/ml, min-max: 0.9-102 ng/ml) compared to patients homozygous for the -173*G allele (median: 5.8 ng/ml, min-max: 0.5-25 ng/ml) \( (p = 0.01) \). In contrast, no difference in synovial MIF levels was found between carriers of CATT\textsubscript{(5,6)} (median: 7.2 ng/ml, min-max: 0.5-100 ng/ml) and carriers of CATT\textsubscript{(6,7)} (median: 9.25 ng/ml, min-max: 0.9-45 ng/ml) \( (p = 0.15) \), nor between carriers of CATT\textsubscript{(6)} (median: 9 ng/ml, min-max: 0.8-27 ng/ml) and carriers of CATT\textsubscript{(6,5)} (median: 7.2 ng/ml, min-max: 0.5-102 ng/ml) \( (p = 1) \). 

**Response to intra-articular glucocorticoid therapy and MIF promoter polymorphisms**

Response to intra-articular glucocorticoid therapy was assessed by comparing the number of months of articular remission achieved following injection with TXA. Dividing patients based on their MIF -173 genotype, we found that carriers of the -173*C allele \( (n = 40, \text{injection number} = 200, \text{median: 7 months, min-max: 1-39 months}) \) had a significantly shorter length of clinical remission following intra-articular glucocorticoid injection compared to patients homozygous for MIF -173*G allele \( (n = 40, \text{injection number} = 200, \text{median: 10 months, min-max: 2-66 months}) \) \( (p < 0.001) \). Carriage of the MIF-173*C allele led to a 46% reduction in the time a joint spent in remission following intra-articular glucocorticoid injection compared to patients with a CATT\textsubscript{(6,7)} carrier \( (n = 40, \text{injection number} = 200, \text{median: 10 months, min-max: 2-66 months}) \) \( (p < 0.001) \). Carriage of the MIF-173*C allele had a significantly lower frequency of a joint being approximately 4 times more likely to relapse within the first 3 months \( (\text{odds ratio} = 3.9, 95\% \text{CI: 1.4-10.5}, p = 0.007) \). There was no significant difference between the CATT genotypes and the duration of remission \( (p = 0.3 \text{ for CATT}\textsubscript{(5,6)} \text{vs. CATT}\textsubscript{(7)}, p = 0.26 \text{ for CATT}\textsubscript{(5)} \text{vs. CATT}\textsubscript{(6,7)}) \). 

To further analyze the association between the -173 MIF alleles and the response to intra-articular TXA, injections were divided based on length of clinical remission in 3 groups: short-term response \( (\leq 5 \text{ months, injection number} = 83) \), intermediate response \( (6-15 \text{ months, injection number} = 128) \) and sustained response \( (\geq 16 \text{ months, injection number} = 75) \). Analyzing the -173 MIF allele frequencies in these 3 groups, we found a significant association between the -173*C allele and the length of response to intra-articular TXA \( (p < 0.001 \text{ by Chi square for trend test}) \), with the group with sustained response having a markedly lower frequency of the C allele \( (\text{Fig. 2B}) \). The same analysis performed on the CATT\textsubscript{(5,8)} genotypes yielded no significant result \( (\text{data not shown}) \). These data suggest that the MIF -173*G/C polymorphism is associated with the duration of response to intra-articular injection of glucocorticoids in patients with o-JIA.

**Outcome of o-JIA and MIF promoter polymorphisms**

To analyze the correlation between MIF promoter polymorphisms and outcome of o-JIA, we analyzed data from the 63 patients with a follow-up of 5
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Discussion

In this study, we found that in patients with o-JIA the MIF -173 G/C polymorphism is associated with SF levels of MIF and with the duration of clinical response to intra-articular glucocorticoid therapy.

We did not observe a significant association between MIF promoter polymorphisms and serum MIF levels in patients with o-JIA. This is in apparent contrast with our previous findings in s-JIA (28) and with recent findings in adult RA (27). However, carriers of the MIF -173°C allele had serum MIF levels that were 26% higher than those of G/G homozygous o-JIA patients. This suggests a similar trend compared to the s-JIA (28) and RA (27) studies, and the lack of statistical significance could be due to the sample size. On the other hand, in contrast with s-JIA and RA, in o-JIA the pivotal feature of the disease is joint inflammation. Indeed, a significant portion of o-JIA patients has normal CRP during active disease. In o-JIA, circulating MIF may leak from inflamed joints rather than being produced systemically. Therefore, our findings may also reflect a difference in the pathogenesis of this particular disease.

In patients with o-JIA, the MIF -173*C allele was associated with higher SF levels of MIF. More importantly, this allele was associated with a less sustained response to intra-articular glucocorticoid treatment. Carriers of the MIF -173°C allele were approximately 4 times more likely to relapse within the first 3 months after injection. As we had no C/C allele carriers in our o-JIA population, it was not possible to establish whether the C/C genotype shows an additive effect on the in vivo expression of synovial MIF or on the response to intra-articular glucocorticoid treatment.

In adult RA, two studies related the CATT VNTR to disease susceptibility (35) and severity (36). Moreover, linkage to a 2-locus MIF promoter haplotype, CATT-CATT, was reported in JIA (32) and, recently, the same locus has been shown to be associated with susceptibility to psoriasis (37). In this study, we did not find any significant relation of the MIF CATT5 vs. CATT6, or the MIF CATT5 vs. CATT6.

or more years. Duration of follow-up, age at disease onset, and time lag from disease onset to the time of CHAQ completion were comparable among patients carrying different MIF alleles (Table II). No association was found between polyarticular extension, level of disability at the last follow-up visit measured by CHAQ or occurrence of chronic anterior uveitis and either the -173 G/C or the -794 CAAT7 polymorphisms (Table III). These observations indicate that the MIF promoter polymorphisms may not predict either the severity of articular outcome or the development of chronic anterior uveitis, which is the distinctive extra-articular feature of o-JIA.
alleles with both MIF synovial levels and response to intra-articular glucocorticoid injections. In our population, analysis by haplotype suggested that the CATT -MIF -173*C and the CATT<sub>c</sub>-MIF -173*C haplotypes were associated with reduced duration of remission in response to intra-articular TXA when compared to all the other haplotypes combined (data not shown). However, given all the possible haplotype combinations, the number of patients required to draw clinically relevant conclusions would be too high to collect.

Recently, a study by Radstake showed that, in RA, both the -173 SNP and the -794 CATT repeat were related to radiologic progression as independent variables, therefore implying that the two polymorphisms may act in distinct manners (27). Our data in o-JIA suggest a clinical correlate for the -173 G/C SNP (26, 28). Our previous data in s-JIA (28) and the recent finding of an association with radiologic progression in RA (27) suggest a possible prognostic role of MIF promoter polymorphisms. In this study, we did not find significant associations with disease outcome at 5 years. In this respect, further studies in o-JIA on the association with radiological progression are of interest.

Our results indicate that in o-JIA the MIF -173*C allele is a single marker identifying patients that are prone to a poorer response to intra-articular glucocorticoid therapy. This observation is consistent with the ability of MIF to inhibit the immunosuppressive and anti-inflammatory effects of glucocorticoids (8) and with the known functional relevance of the -173 G/C SNP (26, 28). Our finding that the MIF -173*C allele is associated with high SF MIF levels in o-JIA adds further evidence to the functional in vivo relevance of this polymorphism. A potential practical implication is that early identification of carriers of the MIF -173*C allele may help to identify those patients who deserve administration of higher doses of intra-articular glucocorticoids or early introduction of second-line medications. Notably, preliminary data in a JIA population treated with TXA suggest a correlation between the dose of intra-articular glucocorticoids and the duration of clinical remission (38). In addition, carriers of the MIF -173*C allele may benefit from therapeutic inhibition of MIF. This approach, which is validated by data obtained in animal models of arthritis, is currently being investigated and holds promise (8, 39).

Table II. Demographics of oligo-JIA patients with a follow-up > 5 years divided based on -173 MIF genotype (G/G carriers vs. -173*C allele carriers) or on -794 MIF genotype (CATT<sub>5</sub> vs. CATT<sub>7</sub> and CATT<sub>5</sub> vs. CATT<sub>7</sub>). Data are shown as median (min-max). P values were calculated using the Mann-Whitney U test.

<table>
<thead>
<tr>
<th>-173 MIF Genotype</th>
<th>-794 MIF Genotype</th>
<th>-794 MIF Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G (n = 46)</td>
<td>G/C (n = 17)</td>
<td>G/C (n = 17)</td>
</tr>
<tr>
<td>Age at onset, years</td>
<td>2.3 (1-13)</td>
<td>2.6 (1-12.3)</td>
</tr>
<tr>
<td>Follow-up, years</td>
<td>7.3 (5-22)</td>
<td>8.2 (5-20)</td>
</tr>
<tr>
<td>Time of CHAQ from disease onset, years</td>
<td>7.3 (5-22)</td>
<td>7.6 (5-18.5)</td>
</tr>
</tbody>
</table>

Table III. Extension of joint involvement, CHAQ score, development of chronic uveitis in patients with oligoarticular JIA, divided into two groups based on -173 MIF genotype (G/G carriers vs. -173*C allele carriers) or on -794 MIF genotype (CATT<sub>5</sub> vs. CATT<sub>7</sub> and CATT<sub>5</sub> vs. CATT<sub>7</sub>).

<table>
<thead>
<tr>
<th>-173 MIF Genotype</th>
<th>-794 MIF Genotype</th>
<th>-794 MIF Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G (n = 46)</td>
<td>G/C (n = 17)</td>
<td>G/C (n = 17)</td>
</tr>
<tr>
<td>Patients with extended o-JIA (%)</td>
<td>17 (37%)</td>
<td>11 (65%)</td>
</tr>
<tr>
<td>Median CHAQ at last visit (min-max)</td>
<td>0 (0-1.6)</td>
<td>0.1 (0-1.3)</td>
</tr>
<tr>
<td>CHAQ ≥ 0.75 (%)</td>
<td>6 (13%)</td>
<td>4 (23.5%)</td>
</tr>
<tr>
<td>Patients with chronic uveitis (%)</td>
<td>17 (37%)</td>
<td>6 (35%)</td>
</tr>
</tbody>
</table>

(*) Fisher’s exact test.
(†) Mann-Whitney U test.
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