

# Tumor Necrosis Factor- $\alpha$ Gene Polymorphism in Severe and Mild-Moderate Rheumatoid Arthritis

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**ABSTRACT. Objective.** To examine whether severe rheumatoid arthritis (RA) carries a -238 or +489 tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) genotype different from mild-moderate RA.

**Methods.** We investigated 163 patients (66 with severe disease) and 67 healthy blood donor controls. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism.

**Results.** Patients with severe RA (all active disease despite disease modifying antirheumatic drug combination therapy) disclosed the -238 GG genotype in 100% of cases versus 92.8% of the mild-moderates and 92.5% of controls (OR 11.7, CI 0.6–216,  $p = 0.03$ ). The +489 AA genotype was seen less often in patients than in controls (OR 4.2, CI 0.97–18.4,  $p = 0.045$ ), and the contribution to this trend appeared predominant in the anti-TNF treated subgroup.

**Conclusion.** The -238 AG genotype was absent in severe RA; in contrast, patients with mild-moderate RA disclosed the same frequency as controls. Thus -238 GG homozygosity is associated with severe RA. The +489 AA genotype might instead protect against worse outcome in RA. (J Rheumatol 2002;29:29–33)

*Key Indexing Terms:*

TUMOR NECROSIS FACTOR- $\alpha$  GENE

DISEASE SUSCEPTIBILITY

RHEUMATOID ARTHRITIS

Better understanding of the biological events occurring in rheumatoid synovial tissue has substantially changed the therapeutic approach to rheumatoid arthritis (RA) in the last 5 years. The first acknowledgment has been that the anchor drug for the disease, methotrexate (MTX), commonly employed in moderately aggressive disease, works satisfactorily in 50–60% of patients for a long period of time<sup>1–3</sup>. The second major advance has been the widely recognized clinical usefulness of combination therapies that can significantly help patients that respond poorly to monotherapy with MTX<sup>4,5</sup>. The third crucial advance has been the seminal work of the Kennedy Institute that disclosed the key role played by the cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which became the therapeutic target for patients responding poorly to conventional disease modifying antirheumatic drugs (DMARD)<sup>6–8</sup>. The observation that anti-TNF- $\alpha$  therapy retards or even stops the progression of the anatomic damage has changed our views on the inevitable relentless progression that was

thought to occur with the DMARD. The discovery that the anatomic damage may indeed be contained has led rheumatologists to believe that, in patients with a poor prognosis, early intervention with the biologicals should be adopted to achieve clinical and radiological remission<sup>9</sup>. The goal of therapeutic intervention should be to keep the patient in her/his working capacity and avoid the crippling disease<sup>10</sup>. In the presence of sure prognostic factors, the patient should be treated early, very aggressively.

Unfortunately, we have only a few such well defined prognostic measures and this hinders our capacity to identify these patients at onset. Since TNF- $\alpha$  is the key cytokine in RA, we reassessed the role of TNF- $\alpha$  gene polymorphisms as prognostic markers, taking advantage of a series of patients with severe aggressive disease<sup>11,12</sup> who were treated with anti-TNF- $\alpha$  therapy because of their poor response to a DMARD combination strategy. These patients present the most severe RA and should be the best candidates to reveal data on prognostic features, including the genetic setting. The phenotypes of these patients were compared to those of a cohort of patients who had a good response to MTX, who were classified as having mild-moderate disease.

## MATERIALS AND METHODS

**Subjects.** One hundred sixty-three patients with RA and 67 sex and age matched healthy blood donor controls entered the study. RA was diagnosed on the basis of the American College of Rheumatology 1987 criteria<sup>13</sup>. RA patients were split into 2 subgroups. Group A (97 patients) comprised those in stable partial remission for at least 6 months after MTX treatment at a weekly dose of 15 mg (range 10–25 mg). Stable partial remission was defined<sup>14,15</sup> when patients had < 3 swollen joints and morning stiffness < 30 min. This group was defined as MTX responders (MTX-R). Group B (66

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patients) comprised those who still had active disease despite 6 months of combination therapy [MTX + sulfasalazine (SSZ) + hydroxychloroquine (OHC)<sup>4</sup> or MTX + cyclosporin A (CSA)<sup>5</sup>, including low dose prednisone, 5 mg/day]. Active disease was defined when patients had persistently > 6 swollen joints and morning stiffness  $\geq$  60 min. This group received anti-TNF- $\alpha$  therapy (infliximab according to the schedule of the ATTRACT trial<sup>7</sup>) and was denoted anti-TNF-treated (TNF-T). Demographic and clinical features for the 2 RA subgroups are given in Table 1.

**DNA isolation and genotyping.** DNA was extracted from 7 ml of uncoagulated blood by the "salting out" method, amplified by polymerase chain reaction and analyzed by specific restriction fragment length polymorphism following described procedures<sup>16-18</sup> (Table 2). Each sample was genotyped by comparing the digested PCR products with the known cleavage patterns (Figures 1 and 2).

**Statistics.** Statistical analyses (odds ratio, 95% confidence interval) were performed using Prism software (Graph-Pad, San Diego, CA, USA). We calculated p values by the Yates continuity corrected chi-square test. Since previous studies indicated a decrease of the -238 A allele and the +489 A allele in RA patients, one sided statistical analyses were used to assess whether this allele was decreased.

## RESULTS

**-238 genotyping.** The distribution of the -238 genotypes in our RA population as a whole, in the 2 RA subgroups, and in controls is shown in Table 3.

MTX-R had an overall frequency of GG homozygosity for the -238 polymorphism of 92.8% vs 92.5% in controls, while in the TNF-T subgroup the GG genotype had a frequency of 100% (OR 11.7, CI 0.6-216,  $p = 0.03$  vs controls). Thus in the TNF-T subgroup the AG genotype is lacking, while MTX-R patients disclose a frequency comparable to that of controls. It seems that the slight reduction in AG frequency in the total RA population (4.3%) vs controls (7.5%) reported in other series<sup>19</sup> has to be ascribed to the patients classified as those with severe, nonresponsive RA.

**+489 genotyping.** Table 4 shows the distribution of the +489 genotypes that we studied in a smaller RA population (all the

66 TNF-T and 72 consecutive MTX-R) and in 58 consecutive controls. As shown, there is no substantial difference in the frequencies of the GG/GA genotypes among the 3 popula-

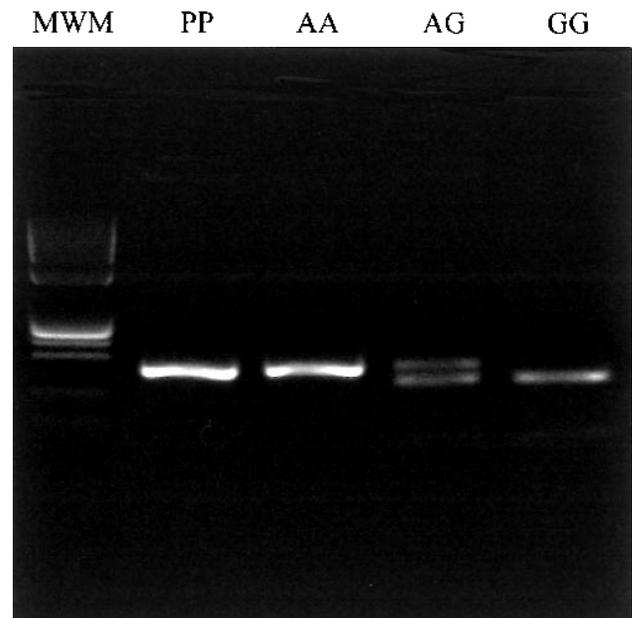


Figure 1. -238 TNF- $\alpha$  polymorphism genotyping through Bam HI digestion of the PCR product (PP = 165 bp). MWM: molecular weight marker, phiX Hae III.

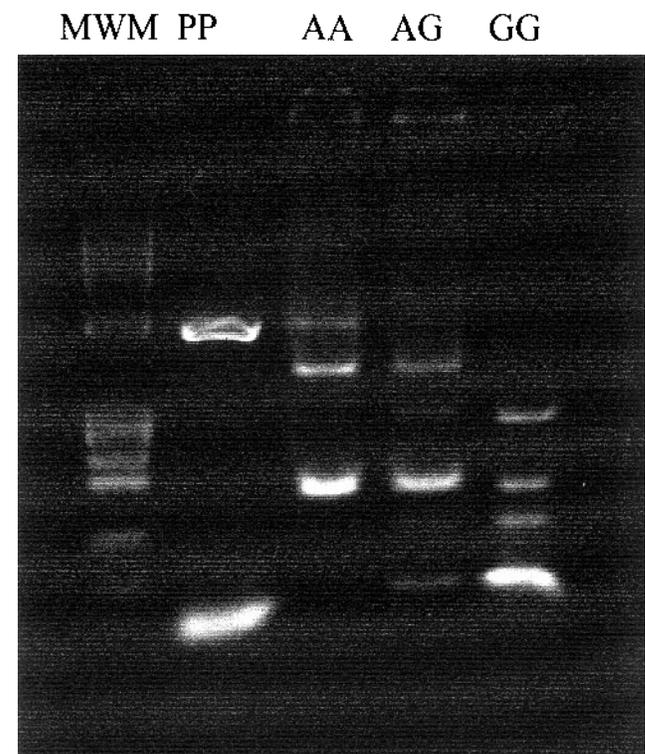


Figure 2. +489 TNF- $\alpha$  polymorphism genotyping through Tai I digestion of the PCR product (PP = 551 bp). MWM: molecular weight marker, phiX Hae III.

Table 1. Demographic and clinical characteristics of nonresponder (TNF-T) and responder (MTX-R) patients with RA.

Variable	TNF- T	MTX-R
No. of patients	66	97
Age, yrs*	53 (27-79)	61 (17-81)
Sex, % female	97	85
Age at onset, yrs*	36 (14-70)	49 (13-76)
Disease duration, yrs*	12 (2-40)	8 (1-51)
Swollen joint count*	10 (6-55)	2 (0-3)
Tender joint count*	13 (1-68)	3 (0-51)
RF positivity, %	70	54
CRP, mg/l*	22.5 (1-137)	4.8 (0.3-50)
Steinbrocker functional class		
I	22.4%	69.1%
II	44.4%	28.4%
III	27%	2.5%
IV	3.2%	-

\* Median (range) RF: rheumatoid factor. CRP: C-reactive protein (normal < 5 mg/l).

Table 2. PCR-RFLP experimental conditions.

Polymorphism	- 238 <sup>16</sup>	+ 489 <sup>17</sup>
Sense primer	5'- AAA CAG ACC ACA GAC CTG GTC-3'	5'-GGA GAG AAG CAA CTA CAG AC-3'
Antisense primer	5' AAG GAT ACC CCT CAC ACT CCC CAT CCT CCC GGA TC-3'	5'-CAC ACT TAG TGA GCA CCT TC-3'
PCR amplification program	95°C × 40 s 61°C × 1 min 72°C × 40 s 40 cycles	95°C × 40 s 60°C × 1 min 72°C × 1 min 40 cycles
Restriction enzyme	Bam HI (37°C)	Tai I (65°C)
Digestion pattern	GG: 123 + 42 bp AG: 165 + 123 + 42 bp AA: 165 bp	GG: 281 + 159 + 111 bp AG: 392 + 281 + 159 + 111 bp AA: 392 + 159 bp

Table 3. -238 TNF- $\alpha$  polymorphism. Genotype and allele frequencies in controls and in the 2 RA subgroups. Odds ratios (OR), confidence intervals (CI, 95%), and p values (Yates continuity corrected chi-square test\*, one-sided\*\*) regarding AG genotype frequency in RA subgroups versus healthy controls are reported.

	GG, %	AG, %	AA	A Allele, %	G Allele, %	n	OR	95% CI	p
Controls	92.5 (62)	7.5 (5)	—	3.8	96.2	67			
MTX-R	92.8 (90)	7.2 (7)	—	3.6	96.4	97	1.03	0.3–3.4	NS
TNF-T	100 (66)	— (0)	—	—	100	66	11.7	0.6–216	0.03
RA total	95.7 (156)	4.3 (7)	—	2.2	97.8	163	1.8	0.5–5.8	NS

\*The Yates correction makes the approximate results from a chi-square test more accurate with small samples.

\*\*One-sided p value is accepted when previous data have indicated that a difference, if any, can only go in one direction. Since all previous series indicated a reduction of A allele in severe RA, we expected to find only a reduction and we therefore chose the one-sided p value. The 2-sided option gives p = 0.07.

Table 4. +489 TNF- $\alpha$  polymorphism. Genotype and allele frequencies in controls and in the 2 RA subgroups. Odds ratios (OR), confidence intervals (CI, 95%), and p values (Yates continuity corrected chi-square test, one-sided) regarding the AA genotype frequency in RA subgroups versus healthy controls are reported.

	GG, %	AG, %	AA, %	A Allele, %	G Allele, %	n	OR	95% CI	p
Controls	69 (40)	22.4 (13)	8.6 (5)	19.8	80.2	58			
MTX-R	72.2 (52)	25 (18)	2.8 (2)	15.3	84.7	72	3.3	0.6–17.7	NS
TNF-T	68.2 (45)	30.3 (20)	1.5 (1)	16.7	83.3	66	6.1	0.7–54.1	0.07
RA total	70.3 (97)	27.5 (38)	2.2 (3)	15.9	84.1	138	4.2	0.97–18.4	0.045

tions. Interestingly, however, we find a much lower frequency of the AA genotype in the RA population in general (OR 4.2, CI 0.97–18.4, p = 0.045) and in the TNF-T subgroup in particular (OR 6.1, CI 0.7–54.1, p = 0.07).

## DISCUSSION

Patients undergoing anti-TNF- $\alpha$  treatment in this study were those who had no response to combination therapies (MTX + CSA or MTX + SSZ + OHC, with optional 5 mg/day prednisone) for at least 6 months. Despite the aggressive treatment

all had persistently active synovitis (> 6 swollen joints, elevated C-reactive protein, CRP). These patients, with active disease despite combination therapies, certainly had the aggressive form of the disease. It is recognized that patients unresponsive to combination therapies with a persistently high number of swollen joints and high CRP represent those progressing to an erosive outcome. Arbitrarily, we defined these patients as the subset with severe RA. On the other hand, as illustrated by the data in Table 1, the MTX-R group represents standard patients with a mild-moderate course (quite

normal CRP, < 3 swollen joints) that had responded well to weekly MTX<sup>15</sup>. Thus the 2 subgroups may be viewed as the 2 faces of RA — the aggressive-progressive and the mild-moderate subtypes<sup>14</sup>.

Since TNF- $\alpha$  is considered the key cytokine in the whole biological process and since TNF- $\alpha$  gene polymorphism has revealed some interesting associations<sup>16-19</sup>, we thought the 2 subgroups represented ideal populations to investigate a possible predisposing role of TNF- $\alpha$  gene polymorphisms toward a more aggressive-progressive phenotype. The Leiden group has shown that the -238 GG genotype is associated with a more severe radiological course<sup>16</sup>, and that even the +489 GG genotype discloses an association with a worse outcome<sup>17</sup>. In our study we certainly confirm these results. Indeed, the -238 GG homozygosity marks all our patients who received anti-TNF- $\alpha$  therapy. Since the frequency of this genotype is still very high in controls (as reported also in the Leiden population<sup>17</sup>), the practical usefulness of this marker must be viewed with caution. Yet the results strongly suggest that the GG genotype does play an important role in predicting an unfavorable course in RA. On the other hand, the AG genotype, not found in patients treated with anti-TNF- $\alpha$  therapy, might exert a protective role against aggressiveness and progression in RA. As for the +489 genotype, we found AA homozygosity much lower in RA patients than in controls and this difference seems to be more imputable to the severe-aggressive cases. The Leiden group has hypothesized a protective role of the A allele in RA<sup>17</sup>. With respect to the North European RA population tested by Brinkman, *et al*<sup>19</sup>, our global RA population disclosed a higher +489 A allele frequency (15.9% vs 6.8%), but if we compare each RA group with its control population (19.8% and 11.8%, respectively), we find comparable differences. In conclusion, even considering the genetic difference between Italians and North Europeans, our data surely confirm the Leiden group's findings and support the hypothesis that the +489 A allele could be in linkage disequilibrium with a genetic setting that may offer a less aggressive phenotype in RA. The biological significance of these findings is far from clear.

The position -238 is located within a Y-box-like sequence<sup>20</sup>, typically found in the regulatory region of the MHC II genes<sup>21</sup>. The Y-box motif is the target sequence of a heteromeric ubiquitous transcription factor called NF-Y<sup>22</sup>, which is unable on its own to activate transcription, but that increases the activity of neighboring enhancer motifs and contributes to the correct positioning of other transcription factors. Interestingly, NF-Y is involved in monocyte to macrophage differentiation<sup>23</sup>, and it has been reported that a polymorphism that alters the Y-box sequence in the HLA-DRB1 promoter affects nuclear protein interaction and transcriptional activation of the gene<sup>21</sup>. Fong and colleagues<sup>24</sup> identified a repressor site involved in the transcriptional regulation of the human TNF- $\alpha$  gene, between the -254 and -230 nucleotides, exactly where the -238 polymorphism maps, but

the transcription factor responsible for this activity is still unknown.

A Canadian group found that 50% of RA patients did not express the NF-Y transcription factor<sup>22</sup>, which binds to DRB promoters and plays a dominant role on the level of the expression of such genes. They hypothesized that the lack of NF-Y protein would result in enhanced expression of DR genes, which carry an inverted CCAAT sequence in their Y boxes. If we suppose that the -238 position is involved in interaction with a negative transcription regulator, the transition from G to A might positively alter this interaction and predispose to better regulation of gene expression. Or the G allele might permit better interaction with a positive regulator of TNF- $\alpha$  expression. Moreover, it is probable that this genetic predisposing setting associates with the absence or presence or misregulated expression of a partner nuclear protein or nucleotide sequence in order to determine the more aggressive phenotype in RA. Recently it was reported that for proper regulation of TNF- $\alpha$  expression, cooperation between the 5'- and 3'-UTR homologous sequences of the gene seems crucial<sup>25</sup>. This latter finding might also explain, at least partially, the lack of concordance among the various promoter/reporter gene experiments to determine the different contribution of the polymorphic allelic variants to the level of TNF- $\alpha$  expression<sup>16,18,26,27</sup>. Thus the hypothesis that mononuclear cells of patients with the -238 GG genotype and/or without the +489 A allele might express more protein, allowing more inflammation, appears quite realistic, but remains to be proven. Work is in progress in this regard. We believe that our data may offer a piece of molecular evidence in the identification of a set of prognostic factors (clinical, biological, and molecular) that might be used to more readily target patients progressing to a worse outcome. These patients might be indicated to receive the most effective, more expensive, anti-TNF- $\alpha$  treatment as soon as possible.

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