

Tumor Necrosis Factor- α –308 Genotypes Influence Inflammatory Activity and TNF- α Serum Concentrations in Children with Juvenile Idiopathic Arthritis

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ABSTRACT. Objective. Considering the relevance of tumor necrosis factor- α (TNF- α) in the pathophysiology of juvenile idiopathic arthritis (JIA), it is likely that polymorphisms in its promoter area may be relevant in disease susceptibility and activity. We investigated if clinical measures of JIA activity and TNF- α serum concentrations were associated with TNF- α –308 genotypes.

Methods. Portuguese patients with JIA in 5 pediatric rheumatology centers were recruited consecutively, along with a control group of healthy subjects. Demographic and clinical data and blood samples were collected from each patient. DNA was extracted for analysis of TNF- α gene promoter polymorphisms at position –308 by restriction fragment-length polymorphism.

Results. One hundred fourteen patients and 117 controls were evaluated; 57% of patients presented the oligoarticular subtype, 25% the polyarticular subtype, 8% the systemic subtype, and 9% had enthesitis-related arthritis and 5% psoriatic arthritis. Twenty-four percent of the patients presented the –308 GA/AA genotypes and 76% the –308 GG genotype, similar to findings in controls. Patients with the –308 GA/AA genotype had higher degree of functional impairment, erythrocyte sedimentation rate, 100-mm visual analog scale score for disease activity, and TNF- α levels compared to those with the –308 GG genotype.

Conclusion. TNF- α –308 GA/AA genotypes were found to be related to higher inflammatory activity and worse measures of disease activity in Portuguese patients with JIA. They were not associated with susceptibility to JIA. (First Release Jan 15 2009; J Rheumatol 2009;36:837–42; doi:10.3899/jrheum.080615)

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PROGNOSIS

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Juvenile idiopathic arthritis (JIA) is the most common chronic arthritis in childhood; it comprises a heterogeneous group of syndromes, of which onset occurs before the age of 16 years, with a disease duration greater than 6 weeks¹. The etiology of JIA remains unclear, but some genetic factors acting in concert are believed to predispose to development of the disease^{2,3}. The best-defined genetic associations have been made with human leukocyte antigen (HLA) system genes⁴⁻⁷. Other molecules, such as interleukin 1 (IL-1), IL-6, IL-17, and tumor necrosis factor- α (TNF- α) have also been implicated in the etiopathogenesis of JIA⁸⁻¹².

TNF- α is a cytokine that plays an important role in inflammation, stimulating the production of many other proinflammatory cytokines. TNF- α is involved in the pathogenesis of JIA^{11,13}, and the level of this cytokine in the serum and synovial fluid of patients with JIA has been shown to vary with disease activity^{14,15}. Moreover, some studies have shown that the production of TNF- α is influenced by polymorphisms in its gene promoter region. In particular, the G/A transition at position -308 appears to be crucial for the regulation of TNF- α translation¹⁶⁻¹⁹. Ozen, *et al*²⁰ assessed the TNF- α -308 and -238 polymorphisms in Czech and Turkish patients with JIA and suggested an association between the -308A allele and poor disease outcome in the Turkish group. Zeggini, *et al*²¹ also found a positive association with TNF- α polymorphisms (positions -308A, -238G, +489A, +851A) in a large panel of UK Caucasian patients with oligoarticular JIA. In contrast, Modesto, *et al*²² found no association of 4 TNF- α gene promoter polymorphisms (at positions -376, -308, -238, and -163) with oligoarticular and systemic JIA, in a group of Spanish Caucasian children.

JIA and rheumatoid arthritis (RA) are 2 distinct disease entities, although they share some clinical and pathogenetic factors as well as genetic background²³. Of interest, the -308 TNF- α GA/AA genotypes have been shown to be correlated with disease activity and response to treatment in Portuguese patients with RA^{24,25}. Therefore, our aim was to examine whether clinical, functional, and laboratory measures of JIA activity and serum levels of TNF- α were associated with TNF- α gene promoter polymorphisms at position -308.

MATERIALS AND METHODS

Patients. Patients with the diagnosis of JIA according to the Durban criteria¹ followed in 4 hospitals (Santa Maria Hospital, Egas Moniz Hospital, Garcia de Orta Hospital, and São João Hospital) and one author's private clinic in Portugal were recruited consecutively from March 2005 to May 2007. Patients who did not fulfil all the criteria were excluded. Written informed consent was obtained from all parents and also from patients who were older than 12 years of age. Research was carried out in compliance with the Declaration of Helsinki, and all the ethics committees of the participating hospitals and clinic approved the study.

For each patient, a data collection protocol was applied to evaluate age, sex, weight, height, ethnic origin, disease and followup duration, history of uveitis, family history of rheumatic diseases, number of joints with active

disease and/or limited range of motion, visual analog scale (VAS; 100 mm) score for disease activity, patient's functional status, as assessed by the Portuguese version of the Childhood Health Assessment Questionnaire (CHAQ)²⁶, use of steroids and antiinflammatory drugs, and presence of serum rheumatoid factor (RF) and antinuclear antibodies (ANA). Patients were classified, at 6 months after diagnosis, into one of 7 disease categories, each with its own specific characteristics, as follows: oligoarticular (affecting up to 4 joints during the first 6 months of disease), which can be subdivided into persistent oligoarthritis (up to 4 joints even after the first 6 months of disease) or extended oligoarthritis (affecting ≥ 5 joints after the first 6 months); polyarticular (affecting ≥ 5 joints during the first 6 months of disease), which can be subdivided as RF-positive or RF-negative polyarthritis; and systemic arthritis was defined as arthritis coincident with or preceded by daily fever for at least 2 weeks of duration, accompanied by one or more manifestations (including transient evanescent rash, lymphadenopathy, hepatomegaly or splenomegaly, and serositis). The 2 other JIA categories considered were enthesitis-related arthritis and psoriatic arthritis. Patients whose diagnosis was established in the previous 6 months of protocol application were considered as presenting zero years of disease duration. We also included young adults with active JIA (whose disease was diagnosed before age 16 years).

A blood sample was collected from each patient for determination of erythrocyte sedimentation rate (ESR) and serum TNF- α levels. In patients receiving TNF- α antagonists (etanercept) the blood sample was collected under this therapy. DNA was extracted from blood to determine the genotype at position -308 of the TNF- α gene by restriction fragment-length polymorphism (RFLP). The same polymorphism was also assessed in a sample of healthy controls from the same geographic and ethnic origin as the patients.

DNA analysis and TNF- α assessment. Blood was collected in EDTA-containing tubes and DNA extraction was performed using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations.

TNF- α gene -308 polymorphisms (G/A) were analyzed by RFLP using the forward primer 5'-AAT AGG TTT TGA GGG CCA TG-3' and the reverse primer 5'-ATC TGG AGG AAG CGG TAG TG-3'. The forward primer contained one nucleotide mismatch (underlined above), which allowed use of the restriction enzyme *NcoI* (New England Biolabs, Hitchin, UK) for detection of -308G/A polymorphisms. Polymerase chain reaction was performed in a 50 μ l reaction mixture containing 100 ng genomic DNA, 40 nM of each primer, 0.2 mM of each dNTP, 15 mM of MgCl₂, and 0.4 U of *Taq* DNA polymerase (ABgene, Epsom, UK). The reaction mixture was incubated 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s. Digestion with *NcoI* was performed at 37°C, as described by the manufacturer.

The TNF- α concentration was determined in duplicate in serum samples from patients using the DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA), as recommended by the manufacturer.

Statistical analysis. Continuous variables were described as mean \pm standard deviation with a normal distribution, or median (interquartile range) if otherwise. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare continuous or categorical variables, respectively, among genotype groups. As only one subject was found with the AA genotype, this was grouped with the GA genotype (GA/AA) for statistical analysis. Excluding the subject with AA genotype did not change the conclusions. Statistical tests were considered significant when *p* values were ≤ 0.05 . Statistical analysis was performed using R software²⁷.

RESULTS

One hundred fourteen Portuguese Caucasian patients with JIA were evaluated, 68% were girls, with a mean age of 12.7 \pm 6.5 years. The mean body mass index was 19.0 \pm 3.2 kg/m². The median disease duration was 4 years (range

0–28; zero years in 4 patients whose diagnosis was established in the 6 months before the protocol and 28 years in a 42-year-old patient with symptoms since age 14 years) and the median followup period was 3 years (range 0–28). One hundred seventeen healthy subjects were used as controls. The frequency of –308 TNF- α genotype was similar between patients with JIA and controls (patients 24% GA/AA and 76% GG vs controls 22% GA/AA and 78% GG; $p = 0.876$; Table 1).

Thirty-one patients (27%) had a family member with a rheumatic disease (RA, rheumatic fever, psoriasis, JIA, or ankylosing spondylitis). Forty-one patients (36%) had been treated with oral steroids, 89 (78%) with nonsteroidal anti-inflammatory drugs, 65 (59%) with methotrexate, and 15 (13%) with etanercept.

Sixty-five (57%) patients presented the oligoarticular disease subtype [48 (42%) had the oligoarticular persistent pattern and 17 (15%) developed the oligoarticular extended form], 24 (21%) the polyarticular subtype, 10 (9%) enthesitis-related arthritis, 9 (8%) systemic arthritis subtype, and 6 (5%) psoriatic arthritis subtype.

ANA were detected in 39% of patients with JIA (11 missing results). The –308 genotype frequencies were similar between patients with ANA-positive JIA and ANA-negative JIA (16.0% GA/AA and 84.0% GG vs 24.5% GA/AA and 75.5% GG, respectively). The frequency of ANA was particularly high in the group of patients with the oligoarticular subtype: 49% of tested individuals had this autoantibody in the serum. A lower frequency was observed in the other subtypes: 26.3% in the polyarticular, 20.0% in enthesitis-related arthritis, 14.3% in the systemic, and 25.0% in the psoriatic.

In addition, among patients with the oligoarticular subtype, uveitis occurred in 12 (19%) patients and ANA were detected in 5 (42%) of these patients.

Forty-five percent of patients with polyarticular JIA and 9% of patients with the oligoarticular subtype were RF-positive (5 missing results). No RF was detected in serum of patients with systemic, psoriatic, or enthesitis-related arthritis.

Patients with the oligoarticular persistent subtype presented a trend for a higher frequency of –308 GG genotype (90%) as compared to other JIA subtypes and controls ($p = 0.080$). In contrast, a trend for a higher frequency of the –308 GA/AA genotype was present in patients with the polyarticular (38%; $p = 0.123$) and the psoriatic (50%) subtypes ($p = 0.141$). We observed that the distribution of genotype frequencies of oligoarticular subtype patients (oligoarticular persistent and extended) was strongly associated with the GG genotype, compared with “non-oligoarticular” subtypes (polyarticular, enthesitis-related arthritis, systemic, and psoriatic arthritis; $p = 0.007$; Figure 1).

We also found that patients with the –308 GA/AA polymorphism had significantly higher ESR (28.4 ± 24.2 vs 15.0 ± 11.7 mm/h, respectively; $p = 0.007$) and TNF- α levels (215.1 ± 176.0 vs 96.2 ± 124.2 pg/ml; $p = 0.003$), and a trend to a higher degree of functional impairment, as evaluated by CHAQ (0.46 ± 0.66 vs 0.27 ± 0.52 ; $p = 0.243$) and disease activity VAS (22.3 ± 26.0 vs 13.7 ± 20.3 mm; $p = 0.166$; Figure 2).

Among the 17 patients treated with etanercept, 87% had –308 GG genotype and 13% were –308 GA/AA (not statistically different from the genotype frequency in other patients and controls). Serum TNF- α levels in this group

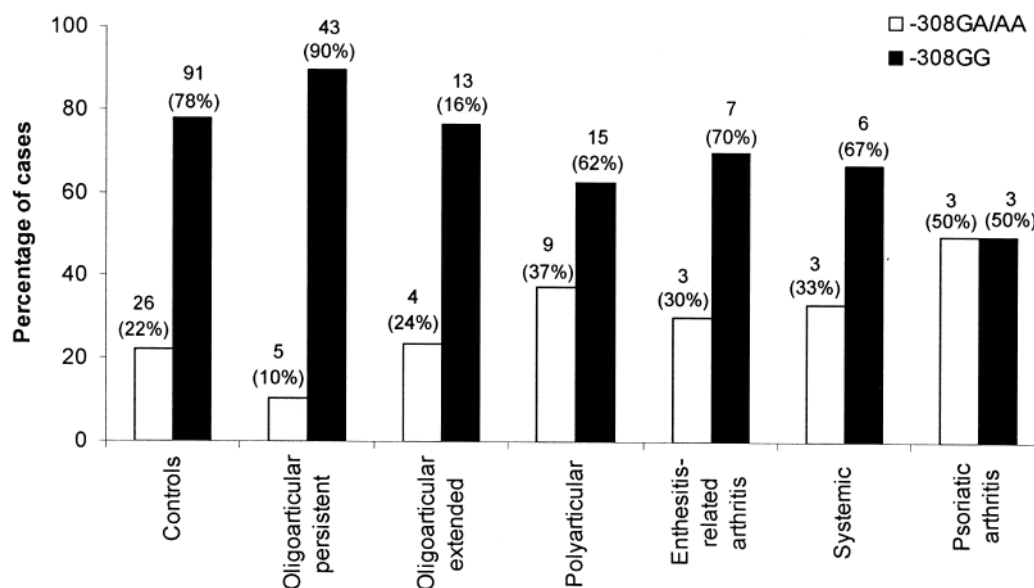


Figure 1. TNF- α promoter –308 genotype frequency in JIA subgroups and controls. No statistically significant differences between patients and controls were observed for –308 genotype ($p = 0.876$). The oligoarticular persistent subtype presented the highest proportion of GG (90%), while GA/AA was significantly increased in the polyarticular (38%) and psoriatic arthritis (50%) groups.

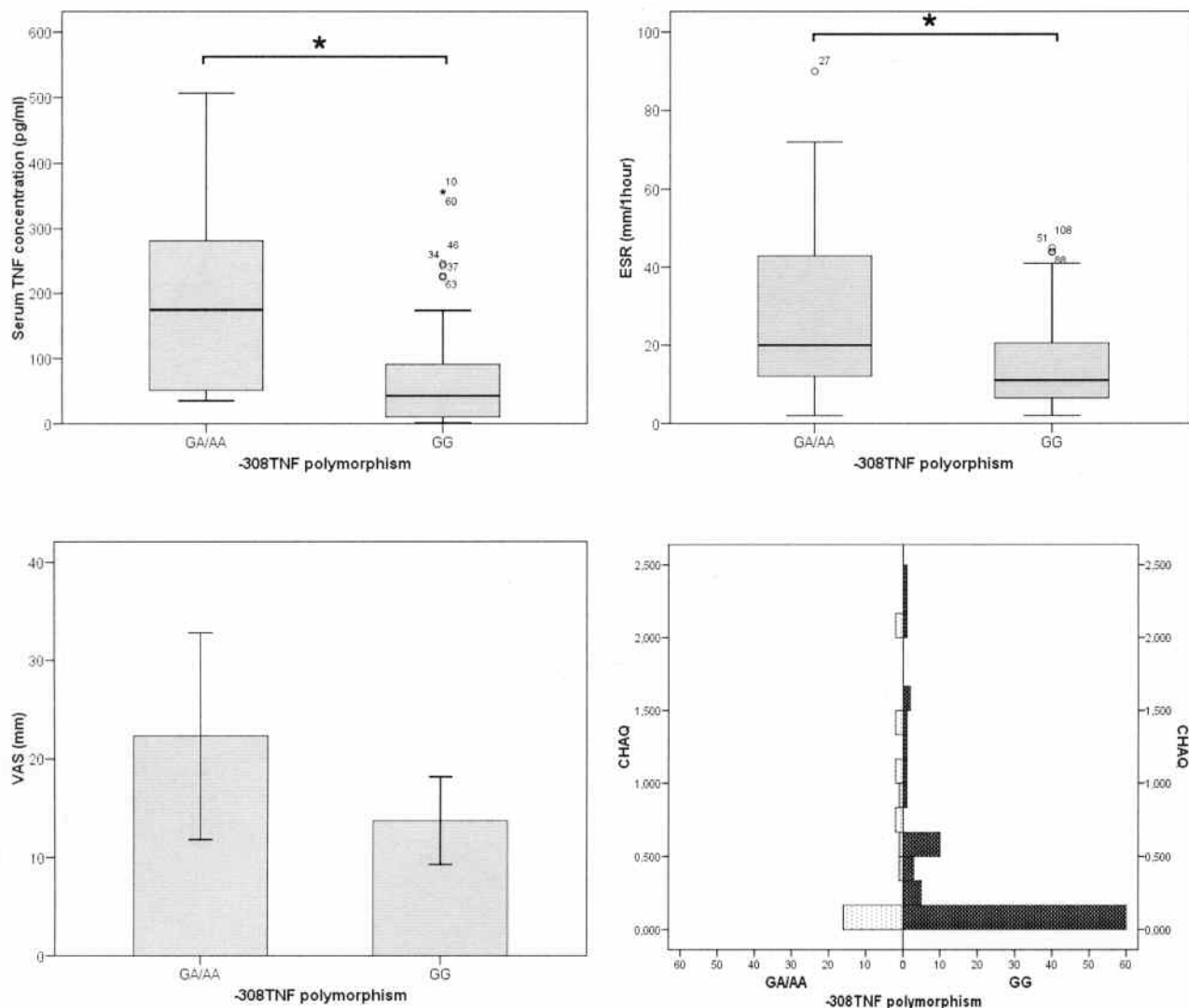


Figure 2. -308 GA/AA was shown to be strongly associated with inflammatory and disease activity indicators. We observed that erythrocyte sedimentation rate (ESR; $p = 0.007$) and serum TNF- α levels ($p = 0.003$) were significantly higher in subjects with -308 GA/AA genotype. CHAQ ($p = 0.243$) and visual analog scale (VAS) scores for disease activity ($p = 0.166$) were higher among subjects with -308 GA/AA (not statistically significant).

were not significantly different from the rest of the study population (114.1 ± 138.5 vs 139.0 ± 200.4 pg/ml; $p = 0.487$).

DISCUSSION

We found that TNF- α -308 genotype frequencies were similar between patients with JIA and controls. Compared to patients with the other JIA subtypes, the oligoarticular subtype (including persistent and extended forms) presented a higher frequency of the GG genotype. Patients with the polyarticular and psoriatic JIA subtypes presented a trend for a higher frequency of -308 GA/AA genotype compared to other subtypes and controls. The presence of the -308A allele was associated with higher level of inflammatory activity, revealed by higher ESR values and serum TNF- α levels ($p < 0.05$), and also with a trend for a lower function-

al capacity and higher disease activity VAS values, although these were not statistically significant. In a previous study our group focused on Portuguese patients with RA²⁵, and we found a positive association between position -308 of the TNF- α gene promoter and work disability and radiologic progression. Our current results are coherent with the data we obtained in that study and reinforce the relevance of -308 polymorphisms in arthritis activity and severity in the Portuguese population.

Fifteen percent of the patients (17 patients) were treated with the TNF- α blocker etanercept. Among these patients, we found no difference in genotype frequency compared to other patients and controls, suggesting that -308 polymorphisms of TNF- α gene do not influence the selection for treatment with biological agents. Serum samples were collected in patients undergoing this therapy, which might have

caused underestimation of the total serum TNF- α concentration. Data on the influence of the polymorphism at position -308 on clinical response to TNF- α antagonists are controversial. In a study from a French group²⁸ performed in 59 patients with RA, those carrying the rare A allele were twice as likely to have no response to infliximab compared to those with the GG genotype of the -308 polymorphism. In another study²⁴, Portuguese RA patients with the -308 GA/AA genotypes presented a worse clinical response to anti-TNF- α therapies and a trend for worse HAQ result. In contrast, Marotte, *et al*²⁹ did not observe any link between the TNF -238 and -308 polymorphisms and joint destruction or selection for infliximab treatment. Similarly, TNF genotypes had no effect on the clinical response to infliximab. Miceli-Richard, *et al*³⁰ also found no association between clinical response to another TNF- α blocker (adalimumab) and any of 3 TNF- α gene promoter polymorphisms (-238, -308, -857) tested individually.

We found an association between the -308 GA/AA polymorphisms and total serum TNF- α concentration. However, in a study from Marotte, *et al*³¹ the -308A allele was associated with higher level of circulating TNF- α bioactivity, but not with protein levels, indicating that endogenous inhibitors must be taken into account. To date, there is no consensus regarding the functional significance of TNF- α gene polymorphisms, and there is no evidence that simple determination of plasma TNF levels allows such prediction³².

In a study involving simplex families consisting of a parent and a child with JIA, as well as healthy individuals, Zeggini, *et al* reported that the -308A allele was associated with oligoarthritis in the whole group and the persistent and extended disease subsets separately²¹. In contrast, in a recent study with a cohort of 107 patients with JIA, Cimaz, *et al*³³ studied TNF- α and IL1 gene polymorphisms, including the TNF- α -308 polymorphism, and no relationship was detected between these polymorphisms and the disease phenotype or response to TNF inhibitors. In accord with that report, we found no association between TNF- α -308 G/A promoter polymorphisms and susceptibility to JIA. Nevertheless, the frequency of the -308 GG genotype was higher in the oligoarticular JIA subtype, compared to the other subtypes, and the -308A allele was associated with higher level of inflammatory activity. In agreement with our observation, in a study performed in Turkish and Czech patients, the -308A allele was significantly associated with a poor outcome in the Turkish group ($p = 0.005$), but there was no association in the Czech patients²⁰.

The relatively small number of patients in some of the disease subgroups may have significantly reduced the power of our study to detect potential differences in allele or genotype frequency between JIA subtypes. Our populations of patients and controls were both from Portugal, with a similar genetic background. However, we acknowledged the lim-

itations of direct comparisons of existing studies due to different ethnic populations and systems of nomenclature and classification. The genetic component of JIA is complex, involving the effects of multiple genes at various points in the disease pathology³. TNF- α is a proinflammatory cytokine implicated in the etiopathogenesis of a broad range of diseases, including JIA. In our study, nearly one-third of the patients had a relative with a rheumatic disease, which reinforces the role of genetic factors in these diseases.

In conclusion, in our study population -308 TNF- α GA/AA genotypes were found to be associated with higher level of inflammatory activity and higher serum concentrations of TNF- α , and the -308 GG genotype was associated with the oligoarticular subtype of the disease. However, polymorphisms in the TNF- α -308 position do not appear to have a relevant role in susceptibility to JIA.

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