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INFLUENCE OF PROMOTER POLYMORPHISMS OF THE TNF-α (-308G/A) AND IL-6 (-174G/C) GENES ON THERAPEUTIC RESPONSE TO ETANERCEPT IN RHEUMATOID ARTHRITIS

UTICAJ POLIMORFIZAMA TNF-α (-308G/A) I IL-6 (-174G/C) GENA NA TERAPIJSKI ODGOVOR NA ETANERCEPT U REUMATOIDNOM ARTRITISU

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Summary

Background: The study was undertaken to assess the influence of functional -308G/A TNF-α (rs 1800629) and -174G/C IL-6 (rs1800795) promoter polymorphisms on the therapeutic response to etanercept, a TNF-α blocker, in patients with rheumatoid arthritis (RA).

Methods: Seventy-three patients suffering from active RA were studied, at baseline and 6 and 12 months after therapy. The therapeutic response was estimated according to the European League Against Rheumatism response criteria. Patients were genotyped for -308G/A TNF-α and -174G/C IL-6 polymorphisms by the PCR-RFLP method, and the influence of genotype on etanercept response was assessed.

Results: No difference in the percentage of responders (patients who had DAS28 improvement > 1.2) between patients with the TNF-α -308GG and GA and AA genotype was detected after 6 and 12 months of treatment. After 12 months of treatment the percentage of responders was significantly increased in patients with the IL-6 -174GG genotype compared with those with the GC or CC genotype (p=0.006 by Chi-square test). Evaluation of the patients

Kratak sadržaj

Uvod: Genetički faktori su značajni za predviđanje ishoda lečenja bolesnika sa reumatoidnim artritisom (RA). Cilj studije bio je da se ispita uticaj -308G/A TNF-α (rs 1800629) i -174G/C IL-6 (rs1800795) promotorskih polimorfizama na terapijski odgovor na etanercept.

Metode: U studiju su bila uključena 73 pacijenta sa aktivnim RA. Terapijski odgovor je procenjivan posle 6 i 12 meseci terapije po kriterijumima Evropske lige protiv reumatizma. Genotipizacija pacijenata za polimorfizme -308G/A TNF-α i -174G/C IL-6 urađena je PCR-RFLP metodom i procenjivan je uticaj genotipova na odgovor na etanercept.

Rezultati: Nije bilo razlike u procentu respondera (pacijenti kod kojih se DAS28 popravio > 1,2) između pacijenata sa TNF-α -308GG, GA i AA genotipom ni posle 6 ni posle 12 meseci tretmana. Posle 12 meseci lečenja procenat respondera je bio značajno veći kod pacijenata sa IL-6 -174GG u odnosu na pacijente sa GC ili CC genotipom (p=0,006, χ^2 test). Poređenje pacijenata prema kombinovanim IL-6/TNF-α genotipovima pokazalo je da je IL-6 -174GG / TNF-α -308GG genotip učestaliji kod respondera u odnosu na druge kombinovane genotipove

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according to their combined IL-6/TNF-α genotypes showed that patients with the IL-6 -174GG / TNF-α -308GG genotype were more frequent among the responders compared to those with other combined genotypes (p=0.022 by Chi-square test). More precisely, all patients with the combined IL-6 -174GG / TNF-α -308GG genotype were responders after 12 months of etanercept treatment.
Conclusions: The study suggests that patients who are genetically low TNF-α and IL-6 producers are the best responders to etanercept therapy.

Keywords: etanercept, pharmacogenetics, rheumatoid arthritis, -308G/A TNF-α gene polymorphism, -174G/C IL-6 gene polymorphism

(p=0.022, χ^2 test). Tačnije, svi pacijenti sa kombinovanim IL-6 -174GG / TNF-α -308GG genotipom bili su responderi posle 12 meseci terapije etanerceptom.
Zaključak: Studija pokazuje da bolesnici sa genotipovima koji se povezuju sa manjom produkcijom TNF-α i IL-6 najbolje odgovaraju na terapiju etanerceptom.

Ključne reči: etanercept, reumatoidni artritis, farmakogenomika, -308G/A TNF-α genski polimorfizam, -174G/C IL-6 genski polimorfizam

Introduction

Anti-TNF therapy has made a significant contribution to the treatment of patients with rheumatoid arthritis (RA). However, these agents are expensive and have significant side effects. Favorable therapeutic response was detected in 50%–70% of patients treated with TNF-α blockers. In the remaining 30%–50% of patients treatment is usually aborted because of the ineffectiveness or side effects of TNF-α inhibitors (1). This is why patient selection is important for effective application of this drug. In addition to clinical parameters, it is necessary to know other predictors of treatment response to anti-TNF-α agents. In recent years, pharmacogenetic research has been focused on finding genetic markers that will be important for forecasting therapeutic outcome of the biological drugs (2).

Considering the central role that TNF-α and IL-6 play in the pathogenesis of RA (3, 4), it is possible that different production of these cytokines may not only affect the natural course of the disease, but also the response to therapy such as TNF-α blockade. Several genetic polymorphisms in the promoter of the IL-6 and TNF-α genes associated with different constitutive and induced production of these cytokines were reported. The genetic variant at position -308G/A (rs 1800629) of the gene encoding TNF-α was found to have functional effects on the gene transcriptional activity. The rare TNF2 allele (-308A) is associated with higher TNF-α production (5). Similarly, the single nucleotide polymorphism (SNP) at position -174G/C of the IL-6 promoter (rs1800795) has been found to alter *in vitro* the transcriptional response to stimuli such as LPS and IL-1 in HeLa cells (6). Carriage of the C allele at this SNP has been shown to correlate with higher serum concentrations of IL-6 in general populations (7) and in RA patients (8).

Several studies and meta-analyses have analysed the association between -308G/A TNF-α and -174G/C IL-6 SNPs and RA activity and response to TNF-α blockers in RA. An association between the -308G/A TNF-α polymorphism and treatment response was found in certain studies (9–14) while two meta-analyses did not confirm this association (15, 16). Also, there have been conflicting reports of the effect of the IL6 -174G/C polymorphism on anti-TNF response in RA. In the study conducted by Cessareli et al. (17), no differences in the proportions of responders between IL-6 -174G/C genotypic groups was demonstrated, while in our previous study (18) and in a meta-analysis (19) combining results from 199 Spanish RA patients with data from our previous study, association between the IL-6 -174G/C polymorphism and the response to TNF-α blockers was found. However, it is known that TNF-α stimulates production of IL-6 (20) and that reduction of IL-6 serum concentration in RA patients treated with TNF-α blockers is a downstream effect (21), suggesting that interactions between TNF-α and IL-6 cytokine genes may influence disease activity and the clinical response to TNF-α blockade. In a previous study, we found that the -174G/C IL-6 gene promoter polymorphism influences the clinical response to etanercept (18). The aim of this study was to examine the possible influence of -308G/A TNF-α polymorphism and its interaction with -174G/C IL-6 polymorphism on the response to etanercept.

Materials and Methods

Patients and treatment protocol

The study was conducted in a cohort of 73 consecutive Caucasian patients with active RA refractory to conventional disease-modifying antirheumatic drugs (DMARDs). All patients who started to receive etanercept therapy in the years 2009 and 2010 were included in the study. Before starting treatment with etanercept, tuberculin skin test and chest X-ray were performed in all patients and both active and inactive ('latent') tuberculosis were excluded. Also, in all patients testing for HBC and HCV infection was negative. The patients fulfilled the 1987 American College of Rheumatology (formerly the American Rheumatism Association) criteria for the classification of RA (22) and were enrolled at the Institute of

Rheumatology, Faculty of Medicine, University of Belgrade, Serbia. The patients were treated with subcutaneous etanercept, 50 mg once per week, in combination with corticosteroids (33 cases) or other DMARDs (methotrexate, 60 cases; sulphasalazine, 3 cases; leflunomide, 5 cases; chloroquine, 5 cases). DMARDs and corticosteroid doses were maintained at a stable concentration during follow-up. The patients were studied before etanercept treatment was started, and six and twelve months after the initiation of etanercept therapy. The clinical status was evaluated by the tender joint count and swollen joint count, Health Assessment Questionnaire (HAQ) score, and the Disease Activity Score in 28 joints (DAS28). Laboratory studies included measurement of serum C-reactive protein (CRP) and rheumatoid factor (RF) and anti-cyclic citrullinated peptides (CCP) antibodies levels. The clinical response was evaluated according to the European League Against Rheumatism (EULAR) response criteria (23). The main outcome was an improvement in the DAS28 score, which is defined as a decrease of at least 1.2.

The study was approved by the Ethics Committee at the Institute of Rheumatology, Faculty of Medicine, Belgrade and was conducted according to the Helsinki Declaration. All patients gave written informed consent to participate in the study.

Genotyping

The genotyping of the -308G/A TNF-α and -174G/C IL-6 gene polymorphisms was performed by a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. Genomic leukocyte DNA was extracted from peripheral blood with a QIAamp DNA Mini Kit (Qiagen).

TNF-α

Primers: *AGGCAATAGGTTTTGAGGGCCAT* (forward) and *ACACTCCCCATCCTCCCTGCT* (reverse). The forward primer sequence was altered (underlined base) to generate a restriction site in the major allele. The conditions for PCR reaction were: a denaturation step at 94 °C for 5 min followed by 35 cycles (94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 45 sec) and final elongation step of 10 min at 72 °C. After digestion with Nco I (MBI Fermentas, Vilnius, Lithuania) fragments of the following length were obtained: for genotype GG – 97+20 bp, GA – 117+97+20 bp and AA – 117 bp.

IL-6

A 525 bp fragment was amplified using the following primers: *GGAGTCACACACTCCACCT* (forward) and *CTGATTGGAAACCTTATTAAG* (reverse). Samples were subjected to a denaturation step at 94 °C for 5 min followed by 35 cycles. Each cycle was as follows: 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 45 sec; the last cycle was followed by a 10 min incubation at 72°C. A 15 μL aliquot of the PCR-product was digested at 37 °C for 2 h with Hin1 II (Fermentas).

Genotyping was performed at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia.

Statistical analysis

Continuous variables were expressed as means ± SD of the values. Differences between the groups were statistically evaluated by the independent sample T-test and One-Way ANOVA followed by the Tukey test for *post hoc* comparisons. Chi-square test or Fisher's exact test were used to test group differences of proportions. Differences were considered to be significant in all cases when $p < 0.05$. Data were analysed using the SPSS for Windows 17.0 software (SPSS, Inc, Chicago, IL, USA).

Results

Baseline characteristics of the patients

The baseline characteristics of 73 patients, before the first injection of etanercept, are summarized in *Table I*. They were 48.10±11.67 (mean±SD) years old, their disease duration was 8.83±7.36 (mean±SD) years and 86.3% were female. All patients had active disease (mean DAS28±SD was 6.10±0.88; mean number of swollen and tender joints±SD was 9.63±4.38 and 12.23±5.36, respectively; mean CRP±SD was 21.15±25.72) and showed functional disability (mean HAQ±SD was 1.06±0.81). Almost half of the patients (43.8%) were rheumatoid factor positive and 75.1% were anti-CCP antibody positive. Also in *Table I*, the baseline characteristics of the patients according to IL-6 -174 and TNF-α-308 genotypic groups were presented. Twenty-three patients had the IL-6 -174GG genotype (31%), 41 (56.2%) had the IL-6 -174GC genotype, and the remaining 9 (12.3%) had the IL-6 -174CC genotype. The TNF-α-308GG genotype was detected in 54 (74%) patients, whereas TNF-α -308GA and AA genotype were found in 18 (24.7%) and 1 (1.4%) patients, respectively, hence the patients with the TNF-α-308GA and AA genotypes were placed in one group. No significant differences were observed between the three IL-6 genotypic groups or between the two TNF-α genotypic groups at baseline with regard to mean age, disease onset, disease duration, RF and anti-CCP antibody status and the disease activity and disability parameters, such as DAS28 and HAQ. A statistically significantly higher number of swollen joints was observed in patients with the -174CC genotype compared with those with GG or GC genotype (CC vs. GG, $p<0.05$; CC vs. GC,

Table I Baseline characteristics of the patients.

Characteristics	All patients (n=73)	IL-6 -174G/C			p value	TNF-α -308G/A		p value
		GG (n=23)	GC (n=41)	CC (n=9)		GG (n=54)	GA and AA (n=19)	
Age, years	48.10±11.67	45.63±11.63	48.8±12.23	51.22±8.63	0.407	48.27±11.63	47.63±12.07	0.839
Women, no. (%)	63 (86.3%)	17 (73.9%)	38 (92.7%)	8 (88.9%)	0.108	47 (87%)	16 (84.2%)	0.714
Disease duration, years	8.83±7.36	8.04±7.74	9.37±7.56	8.33±5.85	0.781	8.44±6.28	9.92±9.91	0.457
Disease onset, years	38.78±13.63	37.93±11.93	39.44±14.42	37.89±15.18	0.899	39.19±12.73	37.66±16.23	0.678
Tender joints, no.	12.23±5.35	11.91±5.32	11.73±5.00	15.33 ± 6.54	0.179	12.65±5.64	11.05±4.39	0.267
Swollen joints, no.	9.63±4.38	9.35±4.73	8.98±3.57	13.33±5.38*a,b	0.022	9.33±4.25	10.47±4.73	0.332
DAS28 score	6.10±0.88	6.14±0.84	6.03±0.91	6.28±0.92	0.716	6.07±0.88	6.19±0.92	0.607
HAQ score	1.06±0.81	1.14±0.98	1.02±0.78	1.06±0.34	0.853	0.95±0.65	1.32±0.92	0.170
Serum CRP, mg/L	21.15±25.72	16.61±22.30	23.07±25.56	24.37±36.23	0.593	18.93±25.04	27.33±27.23	0.225
Positive RF (%)	43.8%	47.8%	41%	62.5%	0.523	47.1%	42.1%	0.711
Positive anti-CCP ab (%)	72.1%	58.3%	75%	85.7%	0.391	78.1%	54.5%	0.241

Values are mean ± SD unless otherwise indicated. DAS28 – Disease Activity Score in 28 joints; HAQ – Health Assessment Questionnaire; CRP – C-reactive protein; RF – rheumatoid factor; anti-CCP ab – anti-cyclic citrullinated peptides antibody. P values were calculated using Chi-square test (for categorical variables) or One-Way ANOVA test and independent samples T-test (for continuous variables); *p<0.05, aCC vs. GG, bCC vs. GC using One-Way ANOVA.

p<0.05). No association between the combined IL-6/TNF-α genotype and baseline demographics and clinical and laboratory features of the patients was detected (data not shown).

-174 IL-6 and -308 TNF-α gene polymorphisms and response to etanercept therapy

Clinical response to etanercept was determined using the DAS28 improvement criteria. Good responders (after 6 and 12 months of treatment) were defined, according to the EULAR criteria, as patients whose DAS28 score improved by at least 1.2 points and bad responders (nonresponders) were defined as patients whose DAS28 score improved by less than 1.2 points (23). By these criteria, of the 73 patients, 62 (84.9%) and 57 (78.1%) were responders after 6 and 12 months of treatment, respectively.

In accordance with our previous study (18), after 12 months of treatment the percentage of responders was significantly increased in patients with the IL-6 -174GG genotype (95.7%) compared with those with the GC (75.6%) or CC (44.4%) genotype (p=0.006 by Chi-square test) (*Table II*). On the other hand, TNF-α genotype did not influence etanercept response, as no significant difference in the percentage of DAS28 responders between patients with TNF-α -308GG and GA/AA genotype was detected after 6 and 12 months of etanercept therapy. When we analyzed patients according to their combined IL-6/TNF-α genotypes, after 12 months of treatment all patients carrying the combined IL-6 -174GG / TNF -α-308GG genotype were responders, whereas the percentage of responders was significantly decreased in patients with other combination of IL-6/TNF-α genotypes (83.3%, 78.6%, 69.2%, 44.4% for patients with IL-6 -174GG / TNF-α -308GA and AA, IL-6 -174GC / TNF-α -308GG, IL-6 -174GC / TNF-α -308GA and AA, IL-6 -174GG / TNF-α -308CC, respectively) (p=0.022 by Chi-square test) (*Table II*).

The mean values of DAS28 score improvement did not significantly differ between IL-6 genotypic groups or between TNF-α genotypic groups (*Table III*). Also, there was no significant difference in the

Table II IL-6 and TNF-α genotypes and etanercept therapy response.

Genotype	Patients, no.	After 6 months of treatment		p value	After 12 months of treatment		p value
		Responders, no. (%)	Nonresponders, no. (%)		Responders,	Nonresponders, no. (%)	
-174 IL-6							
GG	23	20 (87)	3 (13)		22 (95.7)	1 (4.3)	
GC	41	35 (85.4)	6 (14.6)	0.803	31 (75.6)	10 (24.4)	0.006**
CC	9	7 (77.8)	2 (22.2)		4 (44.4)	5 (55.6)	
-308 TNF-α							
GG	54	47 (87)	7 (13)	0.397	43 (79.6)	11 (20.4)	0.590
GA & AA	19	15 (78.9)	4 (21.1)		14 (73.7)	5 (26.3)	
Combined IL-6/TNF-α							
GG/GG	17	16 (94.1)	1 (5.9)		17 (100)	0 (0)	
GG/GA & AA	6	4 (66.7)	2 (33.3)		5 (83.3)	1 (16.7)	
GC/GG	28	24 (85.7)	4 (14.3)	0.548	22 (78.6)	6 (21.4)	0.022*
GC/GA & AA	13	11 (84.6)	2 (15.4)		9 (69.2)	4 (30.8)	
CC/GG	9	7 (77.8)	2 (15.4)		4 (44.4)	5 (55.6)	

P values were calculated using Chi-square test; *p<0.05, **p<0.01.

Table III Improvement in DAS28 scores with etanercept therapy and polymorphisms of IL-6 (-174G/C) and TNF-α (-308G/A).

Genotype	Improvement in DAS28 scores at month 6	p value	Improvement in DAS28 scores at month 12	p value
–174 IL-6				
GG	2.34±1.10			
	2.89±0.98	0.391		0.430
GC	2.00±0.89		2.60±1.36	
CC	1.97±1.23		2.21±0.88	
– 308 TNF-α				
GG	2.20±1.00	0.186	2.59±1.23	0.386
GA & AA	1.84±0.98		2.88±1.16	
Combined IL-6/TNF-α				
GG/GG	2.53±0.98		2.90±1.04	
GG/GA & AA	1.80±1.35		2.86±0.82	
GC/GG	2.06±0.92	0.335	2.47±1.39	0.614
GC/GA & AA	1.86±0.83		2.90±1.31	
CC/GG	1.97±1.23		2.21±0.36	

Values are mean ± SD. DAS28 – Disease Activity Score in 28 joints. P values were calculated using One-Way ANOVA test and independent sample T-test.

magnitude of DAS28 improvement between the combined IL-6/TNF-α genotypes (*Table III*).

Discussion

We analysed the effects of - 308G/A TNF-α and -174G/C IL-6 promoter polymorphisms on the response to etanercept in RA patients with the aim of searching for biomarkers to optimize the use of anti-TNF-α therapy.

Several studies have investigated the relationship between the -308G/A TNF-α polymorphism and disease activity or response to anti-TNF-α treatment in RA patients (9–13). Accordingly (10, 11, 13), in our cohort of RA patients, association between the TNF-α -308G/A polymorphism and clinical manifestations or severity of RA was not found. This could be interpreted either as an absence of or a weak, and insufficient to be detected, influence of this polymorphism on the production of TNF-α. Indeed, it has been reported that other functional SNPs of the TNF-α promoter may interact together with -308G/A polymorphism to determine the overall activity of the TNF-α gene (24). However, although in these previous studies (9, 11–13) an association of TNF-α -308G/A with response to anti-TNF-α therapy was shown, we found no difference in the proportions of responders between TNF-α -308G/A genotypes after 6 and 12 months of etanercept therapy. In agreement with our finding, in a recent meta-analysis (16), which analysed 12 studies plus unpublished data from a total of 426 Dutch patients suffering from RA, association of -308G/A TNF-α polymorphism with different response to anti-TNF-α therapy was not shown. The authors suggested that the relatively small sample size, in previous papers and meta-analyses, is the cause for the association found between -308G/A TNF-α polymorphism and response to TNF-α blockers.

It is known that the systemic effects of etanercept may be the consequence of the crucial role of TNF-α in the complex cytokine network regulating immune and inflammatory responses. Specifically, TNF-α stimulates transcription of the IL-6 gene (25) and induces the secretion of IL-1 and IL-6 (20). Moreover, via IL-12, TNF-α induces the secretion of IFN-γ (26), which is primarily produced by Th1 cells and augments both transcription and mRNA stability of proinflammatory cytokines, including IL-6 in monocytes and macrophages (27). IL-6, IL-1 and IFN-α in turn stimulate the release of TNF-α, thus perpetuating a vicious circle of chronic inflammation (28). Thus, the blockade of TNF-α by etanercept induces decrease in inflammatory cytokine signalling downstream of TNF-α and interrupts a positive feedback loop of TNF-α production. Having all the aforementioned in mind, investigation of the influence of functional genetic polymorphisms of cytokines downstream of TNF-α, such as IL-6, as well as their interactions on the response to etanercept seems to be reasonable.

In accordance with our previous data (18), RA patients with the IL-6 -174GG genotype, known to have lower serum concentrations of IL-6 (8), responded to etanercept better than patients with the GC or CC genotype. When we analyzed patients according to their combined IL-6/TNF-α genotypes, in this study we found that all carriers of the combined IL-6 -174GG / TNF-α -308GG genotype were DAS28 responders after 12 months of therapy. This finding is not surprising since the G allele of -174G/C IL-6 and A allele of -308G/A TNF-α polymorphisms are linked to low and high production of IL-6 and TNF-α, respectively, so in these subjects the lowest secretion of the given cytokines was expected (7, 5). It should be noted that treatment outcome was significantly better in patients with the IL-6 -174GG / TNF-α -308GG combined genotype than in patients carrying at least one allele associated with increased cytokine production. This finding points to the importance of combined, rather than single analysis of the cytokine genotypes to get a more accurate picture of treatment response. Nevertheless, the treatment outcome in the combined IL-6 -174GG / TNF-α -308GG genotype was not statistically better than that obtained after single analysis of the gene encoding IL-6, since the patients with the combined IL-6 -174GG/TNF-α -308GA and AA genotype were highly represented among the responders. Thus, it seems that IL-6 genotyping in RA patients at the onset of the disease could be sufficient to identify suitable candidates for anti-TNF-α blockers.

There is significant variation in allele frequencies among human populations. IL-6 -174G/C polymorphism is the most common in European (35%–50%), less common in Indian (12.6%–14.5%) and the least common in Asian and African populations (close to 0%) (30, 31). TNF-α -308G/A polymorphism is, also, more frequent in European and Indian populations (10%–23%) and less frequent in Asian (2%–9%) and African populations (7%–11%) (29, 30). Therefore, independent pharmacogenetic studies should be performed for the major human populations to determine the influence of IL-6 -174G/C and TNF-α -308 G/A polymorphisms on the efficacy of etanercept.

A limitation of the work is the small sample size. Namely, although etanercept is very effective in treating RA, its cost and strict inclusion criteria are often the main reasons why the published observations in RA patients, as well as our study, generally have a small sample size.

In conclusion, we have shown that genetic variation at the functional -308G/A TNF-α polymorphism does not influence disease activity or response to etanercept therapy in patients with RA. Although the single analysis of the TNF-α genotype did not show a significant influence on the etanercept res-

ponse, analysis of the combined IL-6/TNF-α genotype demonstrated that carriers of the IL-6 -174GG / TNF-α -308GG genotype respond to etanercept better than patients with other combined IL-6/TNF-α genotypes. Since all the carriers of the combined IL-6 -174GG / TNF-α -308GG genotype were responders, it can be assumed that RA patients who are genetically low TNF-α and IL-6 producers may be the most suitable candidates for anti-TNF-α therapy. Validation of these findings in independent cohorts is needed.

Pharmacogenomic testing is currently required before administering maraviroc (for human immunodeficiency virus treatment), cetuximab (for colorectal cancer treatment), trastuzumab (for breast cancer treatment), and dasatinib (for acute lymphoblastic leukemia treatment) (31). In such specific situations, pharmacogenetic testing is done to determine if an individual is likely to benefit from the specific therapeutic agent, rather than to tailor the dosage. Pharmacogenetics in rheumatology is still in its infancy, but it is a fast-growing field. Continued research should soon make individualized drug therapy in the rheumatic diseases a reality (32, 33). Avoiding the pharmacy costs of treatment failures or severe adverse events would be a logical rationale to justify the additional medical costs of this type of testing.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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