

Tumor Necrosis Factor- α Promoter -308/238 Polymorphism Association with Less Severe Disease in Ankylosing Spondylitis is Unrelated to Serum TNF- α and Does Not Predict TNF Inhibitor Response

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ABSTRACT. *Objective.* Despite the clinical efficacy of tumor necrosis factor inhibitors (TNFi), the manner in which TNF- α contributes to disease in patients with ankylosing spondylitis (AS) remains unresolved. We investigated the relationship between TNF- α gene promoter region polymorphism, serum TNF- α levels, and clinical phenotype.

Methods. We did a cross-sectional and longitudinal cohort study in TNFi-naïve patients with AS ($n = 335$). Clinical data and biological samples were collected during a research visit with genotyping for TNF- α -238 A/G and -308 A/G performed by Taqman RT-PCR and TNF levels determined by sandwich ELISA. Longitudinal TNF levels were monitored in unselected patients ($n = 61$).

Results. TNF- α -308 GA/AA genotype was present in 14% and TNF- α -238 GA/AA genotype in 1% of patients. TNF- α -308 GA/AA genotype was associated with a reduced risk of uveitis and better spinal function, while TNF- α -238 GA/AA genotype was associated with later age of onset and lower erythrocyte sedimentation rate (ESR). Serum TNF- α level was lower in patients with AS (151 pg/ml) than in controls (263 pg/ml), because more patients with AS had undetectable serum TNF- α (66 vs 25%, $p < 0.001$). TNFi treatment did not influence serum TNF- α . There was no effect of TNF- α -308/-238 or HLA-B27 genotype on serum TNF- α or subsequent initiation of TNFi.

Conclusion. TNF- α -238 or -308 GA/AA genotypes in patients with AS are associated with signs of less severe disease. Serum TNF- α is, however, undetectable in two-thirds of patients with AS and is not influenced by TNF- α promoter genotype or TNFi therapy. These data suggest a more significant role for TNF- α at local sites of inflammation in AS than through systemic effects. (First Release July 15 2014; J Rheumatol 2014;41:1675-82; doi:10.3899/jrheum.131315)

Key Indexing Terms:

ANKYLOSING SPONDYLITIS
PROMOTER REGION

TUMOR NECROSIS FACTOR
SERUM TUMOR NECROSIS FACTOR

Ankylosing spondylitis (AS) is a chronic inflammatory disease primarily involving the sacroiliac (SI) joints and spine that leads to considerable loss of function as well as an increased risk of premature death^{1,2,3,4}. Subsets of patients develop peripheral arthritis and extraarticular complications including uveitis and heart valve abnormalities. Manage-

ment of the disease changed dramatically with the introduction of tumor necrosis factor (TNF) blocking agents that were shown to lead to both symptomatic and functional improvement⁵. While the antiinflammatory actions of TNF inhibitors clearly indicate a role for TNF- α in AS pathogenesis, the mechanisms by which TNF- α contributes to AS development are unresolved. TNF- α expression is increased in the SI joint, but the source of TNF is unclear⁶. Serum TNF- α levels have been reported as normal, increased, or decreased in patients with AS, while neither serum TNF- α levels nor TNF expression by peripheral T cells correlates well with disease activity^{7,8,9}. HLA-B27 is the strongest genetic factor associated with AS development, but does not explain the clinical heterogeneity of AS in terms of disease severity and response to TNFi development^{10,11}. TNF- α promoter region single-nucleotide polymorphisms (SNP) are not considered independent susceptibility factors for AS, but their modulating effect on TNF- α expression could well be relevant for the phenotypic diversity in AS^{12,13,14,15}. We therefore investigated the relationship between TNF- α -308 and -238 SNP, circulating TNF- α levels, and clinical

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findings including TNFi response in a large cohort of white patients with AS.

MATERIAL AND METHODS

Patients. Patients with AS were selected from an existing disease registry for AS in northern Norway, which has been described in detail previously with all patients fulfilling modified New York criteria³. In our study, we included only patients who attended a research visit during which full blood and serum samples were collected and stored for later analysis. Clinical data were gathered simultaneously with blood collection and included demographic data, questionnaire-based evaluation of function [Bath Ankylosing Spondylitis Functional Index (BASFI)], and complete physical examination, as well as standard laboratory investigations. All patients were TNFi-naïve at the time of inclusion in the study. Followup data were gathered from electronic records with initiation of TNFi therapy as well as the need for TNFi switching registered in a nationwide protocol during the subsequent disease course^{16,17}. The regional ethics committee approved the study protocol and patients gave informed consent.

DNA isolation. Whole blood was collected from patients at the time of study, and DNA was isolated from stored whole blood samples (–20°C) using the Puregene blood core kit A (Qiagen) according to the manufacturers’ protocol. Samples were quantitated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies).

PCR-based low resolution HLA-B27 screening. PCR-based HLA-B27 screening was performed with primers E91s (GGG TCT CAC ACC CTC CAG AAT) and E136as (CGG CGG TCC AGG AGC T) amplifying codons 91–136 of HLA-B27 under the following conditions: 1× Buffer, 0.9 μM of each primer, 0.2 mM dNTP, 0.5 U DynaZyme DNA polymerase, and 200 ng genomic DNA template in a 25 μl reaction. Primers for human growth hormone (HGH.1: TGC CTT CCC AAC CAT TCC CTT A and HGH.2: CCA CTC ACG GAT TTC TGT TGT GTT TC) were used as positive reference-gene.

TNF-α genotyping. Genotyping was performed on DNA from peripheral blood mononuclear cells using commercial Taqman (Applied Biosystems)-based RT-PCR allelic discrimination assays for 2 nonsynonymous SNP in the TNF-α promoter region [rs1800629 (–308 A/G) and rs361525 (–238 A/G)].

For each assay, RT-PCR allele results were confirmed in 9 samples (3 of each genotype) by performing restriction fragment length polymorphism-PCR (RFLP-PCR) using Acl-I enzyme. Direct bidirectional sequencing was then performed to validate the genotyping by PCR-RFLP and reference samples of each genotype were included in each assay. No discrepancies in allele discrimination between RT-PCR and sequencing results were observed.

Serum TNF-α measurement. Serum concentrations for TNF-α were determined in stored serum aliquots (–20°C) by a quantitative sandwich immunoassay technique (Human TNF-alpha DuoSet, R&D Systems) with a lower limit of detection of 16 pg/ml. The manufacturer’s recommendations were followed throughout and results for duplicate runs in each lot averaged. Only sera collected within a 3-day time frame of the date for clinical and biochemical data (n = 260) were included in the association analyses. Sera collected at other timepoints were used in the longitudinal study of TNF-α levels and subsequent anti-TNF therapy. Control sera were obtained from a group of patients (n = 72) with chronic back pain and normal radiographs of the SI joints, normal erythrocyte sedimentation rate (ESR) levels, and negative test for HLA-B27¹⁸.

Statistics. Values are expressed as mean (± SD) unless otherwise indicated. Statistical analyses made use of nonparametric techniques (chi-square, Mann-Whitney U test, Spearman’s rank correlation) because data were often skewed. All calculations were performed using the statistical software package SPSS (version 19.0) and resulting 2-sided p-values corresponding to α levels 5% were considered statistically significant.

RESULTS

Patients included in the genotype study cohort (n = 335) had a mean age of 45 years (± 12.6) at the time of study and a disease duration of 22 years (± 11.2). Overall functional impairment was moderate with a mean BASFI score of 3.5 (± 2.1) and ESR was 19 mm/h (± 12.2). Other patient characteristics at investigation are described in Table 1. Only 5 patients carried the TNF-α –238 A allele (1.5%); they were all heterozygous and all 5 carried the TNF-α –308 GG wild-type genotype. TNF-α –238 A allele presence was associated with later age of onset, lower ESR, and higher Hb levels (Table 2). A total of 47 patients (14%) carried a TNF-α –308 A allele and of these, only 1 patient was homozygous (0.3%). The presence of the TNF-α –308 A allele was associated with a significantly lower risk of uveitis (p = 0.02), better functional test for spinal mobility, and lower ESR (p = 0.09; Table 2). Even though none of the TNF-α –238 A carriers was HLA-B27–negative versus 14% of TNF-α –238 G carriers, there was no statistically significant association between HLA-B27 status and TNF-α –238 or TNF-α –308 genotypes (all p > 0.5).

Overall, serum TNF-α levels in patients with AS (151 pg/ml) were lower in patients with AS than in controls (263 pg/ml; p < 0.001; Figure 1A). This result was strongly influenced by a significantly higher number of patients with AS and undetectable TNF-α levels (68% vs 25% in controls, p < 0.001; Figure 1B). In the remainder of patients with AS, detectable serum TNF-α levels were higher than in controls (472 vs 347 pg/ml, p < 0.01). Association with genotypes was therefore analyzed by an arbitrary serum TNF-α grouping based on SD of mean serum TNF (undetectable: < 16, normal: 16–100, and increased > 100 pg/ml). The distribution of both genotype TNF-α –308 and TNF-α –238 was

Table 1. Characteristics of patients with ankylosing spondylitis (n = 335) included in study.

Characteristics	Mean (SD) or Percent
Age at onset, yrs	23.2 (7.7)
Disease duration, yrs	22.4 (11.2)
Male sex	70
HLA-B27 positive	89
Psoriasis	9
IBD	6
Uveitis	34
BASFI	3.4 (2.1)
Schober’s test, cm	3.5 (1.5)
Chest expansion, cm	4.5 (2.1)
Hemoglobin	13.8 (1.3)
ESR, mm	19.3 (12.2)
WBC, 10 ⁶	7.6 (2.0)
Creatinine, μmol/l	82 (13)
TNF-α, pg/ml	151 (425)

IBD: irritable bowel disease; BASFI: Bath Ankylosing Spondylitis Functional Index; ESR: erythrocyte sedimentation rate; WBC: white blood (cell) count; TNF: tumor necrosis factor.

Table 2. TNF- α promoter allele frequencies and relation to clinical feature. P values are given only for features with $p < 0.1$.

	TNF-238 SNP			TNF-308 SNP		
	A Allele, n = 5	G Allele, n = 330	p	A Allele, n = 47	G Allele, n = 288	p
Age of onset, yrs	31	23	0.06	22.3	23.3	0.82
Age at study, yrs	40.6	45.6	0.49	43.6	45.9	0.36
Male sex, %	76	71	0.91	84.6	89.1	0.47
HLA-B27-positive	100	87	0.9	87	90	0.77
BASFI	2.8	3.4	0.67	3.2	3.4	0.72
Schober's, cm	4.2	3.4	0.43	3.2	3.4	0.21
Chest expansion, cm	5.7	4.3	0.34	5.2	4.3	0.04
Lateral movement, cm	1.7	1.4	0.89	1.7	1.4	0.07
Psoriasis, %	—	10.8	1.0	13.9	10.7	0.57
IBD, %	—	7.7	1.0	5.9	7.9	0.9
Uveitis, %	33	42	0.92	17.4	37.2	0.016
ESR, mm	10	20	0.09	14	20	0.03
Hemoglobin, g/l	15.1	13.8	0.04	14.1	13.8	0.23
Creatinine, μ mol/l	84	82	0.64	84	82	0.16
TNF- α , pg/ml	16	153	0.18	236	137	0.38

TNF: tumor necrosis factor; BASFI: Bath Ankylosing Spondylitis Functional Index; IBD: irritable bowel disease; ESR: erythrocyte sedimentation rate; SNP: single-nucleotide polymorphism.

similar among patients with undetectable, normal, or high serum TNF- α (Figure 2A and 2B). All patients ($n = 4$) carrying TNF- α -238 A were found to have undetectable serum TNF- α versus 68% of TNF- α -238 G carriers ($p = 0.1$). HLA-B27 presence was not associated with serum TNF- α grouping (Figure 2C). Mean serum TNF- α levels also did not differ significantly between the different genotype carriers (data not shown, all $p > 0.2$). Serum TNF- α grouping was unrelated to the ever presence of comorbid conditions, such as psoriasis, inflammatory bowel disease, or uveitis (all $p > 0.2$). Serum TNF- α levels correlated weakly but significantly with serum alkaline phosphatase levels, but not with any other biochemical or clinical marker of disease (Table 3A and 3B).

Treatment data during the subsequent disease course were available from electronic records for 205 patients. During a mean followup of 85 months, TNFi therapy was initiated in 129 patients (63%). Patients who took TNFi were younger at baseline (44 vs 53 yrs, $p = 0.02$), but were similar to patients who were not receiving TNFi with regards to previous disease-modifying antirheumatic drug use (43% vs 57%), baseline CRP (12.7 vs 13.2, $p > 0.3$), ESR (23.6 vs 22.8, $p > 0.6$), TNF- α -238 A (0% vs 2%), and TNF- α -308 A (12% vs 14%) allele frequency as well as TNF- α serum levels (143 vs 126 pg/l; all $p > 0.3$). While 52% of patients were maintained on the initial TNFi, 45% were switched to another TNFi. Their subsequent Bath Ankylosing Spondylitis Disease Activity Index (BASDAI; 2.3 vs 4.6, $p < 0.01$), visual analog scale pain (20 vs 48 mm), and fatigue scores (33 vs 53 mm) remained significantly higher. The frequency of the TNF- α -238 A (0% vs 1%) and TNF- α -308 A allele (13% vs 14%) or initial serum TNF- α levels (68 vs 164 pg/ml) was not associated with achieving an initial BASDAI response (all $p > 0.3$).

The longitudinal course of serum TNF- α levels could be studied in 251 randomly collected serial serum samples from 61 patients (71% male, age 43.8 yrs, and BASFI 5.5 at investigation) during a mean followup of 32 months (median number of samples 3). The number of patients with low/undetectable or high serum TNF- α remained unchanged during TNFi treatment (Figure 3). In patients receiving TNFi therapy, higher maximum levels of TNF- α were seen, but initial and overall mean serum levels were similar between patient groups (Table 4).

DISCUSSION

In our large cohort study of patients with AS, the presence of the non-ancestral A allele in 2 TNF promoter regions was associated with characteristics of less severe disease. However, this was not due to an interaction between genotype and serum TNF- α levels and had little effect on TNFi treatment.

TNF- α promoter region is involved in the regulation of TNF- α expression¹⁹, but polymorphism in this region has not been shown to be an independent risk factor for the development of AS^{20,21,22}. However, AS is a polygenic disease with a diverse clinical phenotype that has been linked to different interactions between several genes^{23,24}. As the obvious clinical efficacy of TNFi treatment in AS is mainly based on empirical evidence, the manner in which TNF- α expression contributes to clinical phenotype remains a pertinent research question^{19,25,26}. In this white AS cohort, the frequency of the minor TNF- α -308 A allele was 7% compared to 17% in the CEU Hapmap population (www.hapmap.org). Several other studies have found a considerably lower prevalence of the non-ancestral TNF A allele in patients with AS, suggesting that this minor A allele may have a protective role against AS^{20,27,28}. Our finding

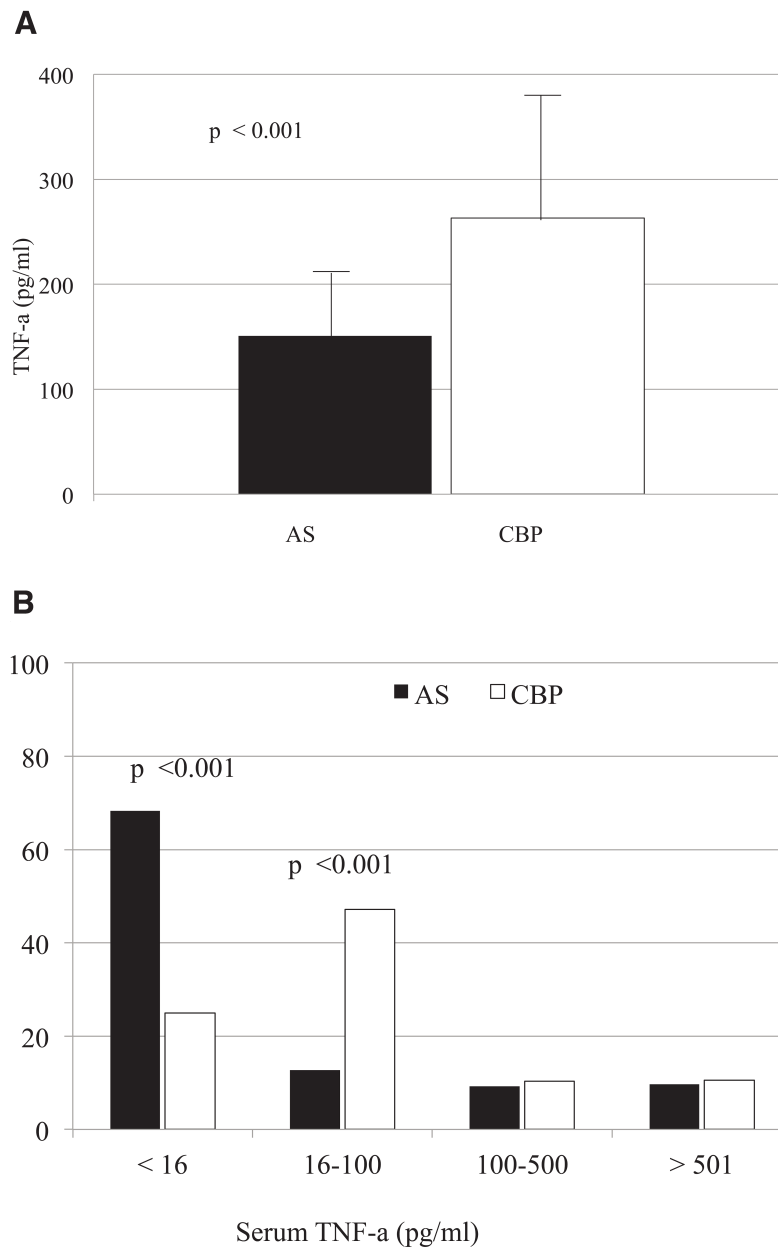


Figure 1. A. Mean TNF- α levels in patients with AS (n = 259) compared to controls (n = 72; by Mann-Whitney U test comparison). Bars represent 2 SD of the mean. B. Distribution of low, normal, and high TNF- α levels in patients with AS and controls (CBP). P value by chi-square analysis. TNF: tumor necrosis factor; AS: ankylosing spondylitis.

that the TNF- α -308 A allele was associated with a lower risk of uveitis, better spinal function, and lower ESR levels supports this theory. Similarly, the prevalence of the TNF- α -238 A allele in this cohort (1.5%) was low^{29,30} and was also associated with lower ESR levels.

The potential benefit of TNF- α -308 or TNF- α -238 A allele carrier status in AS is thought to be the lower expression of the TNF protein with subsequent lower

severity and/or extent of the inflammatory response¹⁹. Overall, TNF- α levels in our AS cohort were low when compared with controls, mainly due to the finding that more than 60% of patients with AS had very low/nondetectable levels and this distribution was significantly different from controls. Such a skewed distribution in patients with AS has been found by other authors as well^{31,32} and that makes it difficult to interpret the usefulness of mean TNF- α serum

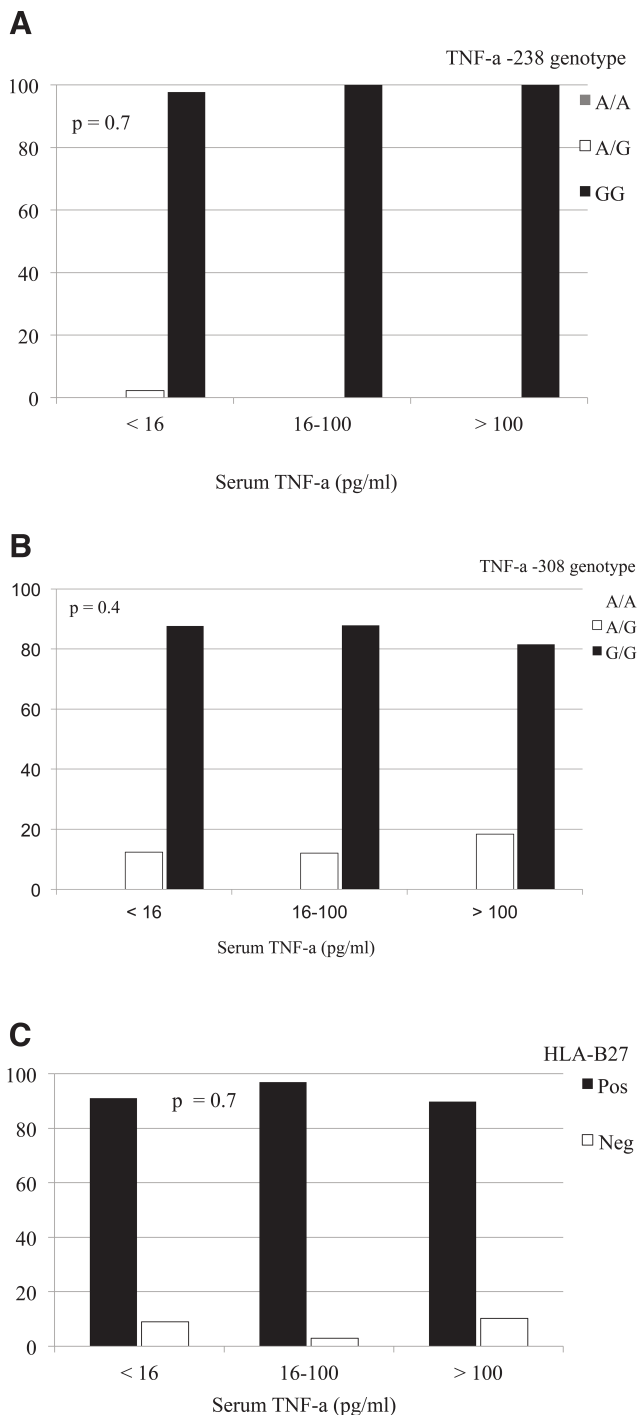


Figure 2. TNF-α -238, TNF-α -308, and HLA-B27 genotype distribution according to serum TNF-α levels in patients with ankylosing spondylitis. TNF: tumor necrosis factor.

levels in the study of AS^{7,9}. In line with the findings on low serum TNF-α, Poddubnyy, *et al*¹⁵ recently expanded on earlier findings of a lower percentage of TNF-α producing CD8+ T cells, especially in HLA-B27 positive patients³³, as

well as lower numbers of TNF-α producing CD4+ cells in patients carrying the TNF-α -308 A allele. It is unknown whether low peripheral TNF-α expression is a normal state in a specific subset of patients with AS or due to successful peripheral mechanisms to control the systemic immune response in AS^{13,34}. In line with earlier studies, we were unable to detect an association between serum TNF-α levels and TNF-α promoter genotypes, although some studies have found TNF promoter polymorphism was associated with changes in mean TNF mRNA expression or serum TNF levels^{20,30,31,32,35}. Given the large number of patients with very low serum TNF-α in this cohort, it was not surprising we were unable to establish an association between serum TNF-α and clinical disease features as in other studies^{7,9}. The significant correlation between serum TNF and levels of alkaline phosphatase is not readily explained. A role for bone-specific alkaline phosphatase in AS bone remodeling has been described, and this may be related to local TNF-α expression^{36,37}. Collectively these data indicate that systemic TNF-α levels are not increased in most patients with AS and that any possible association with genotypic influences will likely need to be further explored at the cellular level at the site of inflammation⁶. Also, many proteins are involved in the TNF pathway and genetic polymorphism in the TNF receptor 1 and TANK-binding kinase protein 1, components of the TNF receptor signaling pathway have been associated with AS susceptibility and may also influence disease severity^{38,39}. We were unable to establish a relationship between TNF-α genotypes or serum levels and the need for and response to TNFi. No other clinical measure than younger age distinguished patients who received TNFi, indicating that age is an important factor in the complex clinical decision to initiate TNFi. Our results confirm the current lack of reliable predictors for TNFi response in patients with inflammatory joint disease^{25,30}, although Seitz, *et al* have reported that while most patients with the TNF-α -308 A allele still achieved BASDAI < 4 scores after 6 months of TNFi as in our dataset, they had a smaller improvement in BASDAI compared to patients carrying TNF-α 308 G allele¹³.

There is little longitudinal data on TNF-α levels in patients with AS. We detected little change in the number of patients with AS and low/undetectable serum TNF-α levels over time, indicating that at the group level the majority of patients with AS remain in a state of low systemic TNF-α. Interestingly, this was seen in both patients taking and not taking TNFi therapy, confirming earlier uncontrolled cohort studies in which treatment with TNFi did not affect already low levels of inflammatory cytokines in small numbers of patient with AS^{38,39}. A recent large cohort study from China reported larger decreases in TNF-α, interleukin 6 (IL-6), IL-17, and IL-23 in patients taking TNFi⁴⁰, but whether this discrepancy is a result of interindividual and intraindividual variations due to genetic differences in TNF-α production is

Table 3. Correlation (Spearman rank coefficients) for association between serum TNF-α levels and clinical findings (A) and biochemical markers (B) in patients with AS.

A	TNF-α	Age	Study	BASFI	Schober	Flexion	Extension	Chest Exp.	Occ-wall	FFD
Age onset	-0.08									
Age study	-0.06									
BASFI	0.04		0.26**							
Schober	-0.06		-0.17**	-0.26**						
Flexion	-0.01		-0.23**	-0.26**	0.63**					
Extension	-0.01		-0.26**	-0.30**	0.50**	0.49**				
Chest exp	0.03		-0.23**	-0.26**	0.31**	0.35**	0.38**			
Occ-wall	0.01		0.24**	0.29**	-0.49**	-0.52**	-0.56**	-0.38**		
FFD	-0.05		0.06	0.30**	-0.35**	-0.29**	-0.31**	-0.26**	0.25**	
Lat. flexion	-0.04		-0.17*	-0.18*	0.33**	0.36**	0.32**	0.25**	-0.33**	-0.19**

B	TNF-α	ALAT	Alb	ALP	ASAT	Hb	Creatinine	ESR	WBC	Platelets	TSH
ALAT	0.05										
Albumen	-0.04	0.17									
ALP	0.15*	0.08	-0.30*								
ASAT	0.02	0.51**	0.17	-0.01							
Hb	-0.01	0.21**	0.29**	-0.09	0.17**						
Creatinine	-0.06	0.10*	0.20	0.02	0.14**	0.21**					
ESR	-0.01	-0.07	-0.38**	0.30**	-0.04	-0.41**	-0.14**				
WBC	-0.03	-0.02	-0.14	0.03	-0.12**	0.01	-0.08	0.12**			
Platelets	0.03	-0.07	-0.23*	0.14*	-0.15**	-0.21**	-0.16**	0.28**	0.20**		
TSH	-0.03	0.13	0.15	0.02	0.08	-0.13	0.02	0.10	-0.06	-0.09	
GT	-0.10	0.42**	-0.01	0.21*	0.19*	0.20*	-0.06	0.11	0.11	0.09	0.32*

* p < 0.05. ** p < 0.01. ALAT: alanine transaminase; Alb: albumen; ALP: alkaline phosphatase; ASAT: aspartate aminotransferase; ESR: erythrocyte sedimentation rate; WBC: white blood (cell) count; TSH: thyroid-stimulating hormone; FFD: finger floor distance; Hb: hemoglobin; GT: glutamyl transferase; TNF: tumor necrosis factor; BASFI: Bath Ankylosing Spondylitis Functional Index.

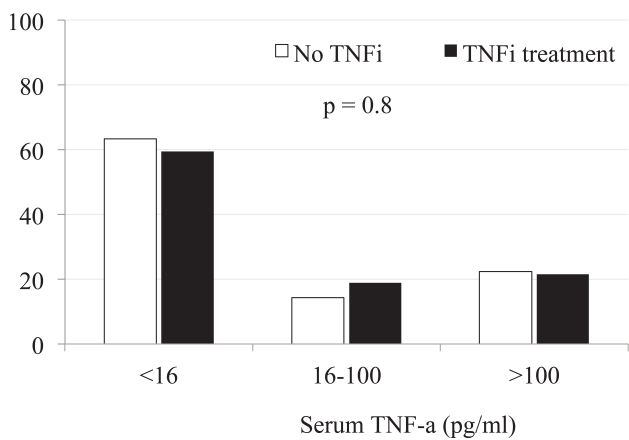


Figure 3. Serum TNF-α levels in patients with ankylosing spondylitis in relation to treatment with TNF-α blocking agents. Y axis indicates percentage of patients. TNF: tumor necrosis factor.

not clear⁴¹. Overall, the data suggest that in white patients with AS, a mechanism may be operative that continuously and successfully suppresses systemic TNF-α production in a large subset of patients over time.

Table 4. Serum TNF level dynamics over time in relation to subsequent TNFi drug treatment in patients with ankylosing spondylitis.

Serum TNF Level, pg/ml	No TNFi, n = 18	TNFi Started, n = 43	p
Initial sTNF-α	111	123	0.2
Mean sTNF-α	160	162	0.06
Max sTNF-α	218	414	0.04
Last sTNF-α	210	101	0.6
Proportion of sera with detectable TNF-α, > 16 pg/ml	25	38	0.2

TNF: tumor necrosis factor; TNFi: TNF inhibitor.

The limitations of our study should be mentioned. While we explored the biologic dogma for TNF-α in AS, other inflammation modulating proteins, such as IL-17, IL-23, and TNFSR1, also play a role in disease expression. We did not study TNF gene expression studies at the cellular levels because mRNA storage was not feasible at the time of data collection. The use of protein serum levels was considered an acceptable practical alternative. Despite the reasonable sample size, the minor allele frequency for the -238 variant was lower than expected (only 5 patients carried the A

allele), making it hard to draw any firm conclusions regarding this SNP. Because 47 patients carried the A allele in the –308 variant, the association between the –308 GA/AA variant with less uveitis and better spinal function is stronger, but will need to be validated in larger studies. Also, results could be biased because both genes are localized in close proximity to the B27 gene in a region with significant linkage disequilibrium (LD). LD analysis (<http://www.broadinstitute.org/mpg/snap/ldsearch.php>) using the Broad SNAP tool indicates an R^2 of 0.009 and D' of 1.00, which makes it unlikely that LD was a significant confounder. Finally, while we failed to establish a link between TNF promoter polymorphism and HLA-B27, this only applies to this white cohort with an almost exclusive presence of the HLA-B27*05 subtype⁴².

The presence of TNF- α –238 and –308 A allele is associated with signs of less severe disease, but this is unrelated to serum TNF- α levels, which are low in the majority of TNFi-naïve white patients with AS. TNF- α genotypes and serum levels have no bearing on the initiation and response to TNFi. These results indicate that TNF- α plays out its main role in AS at local sites of inflammation rather than systemically.

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