Is there a relationship between endothelial nitric oxide synthase gene polymorphisms and ankylosing spondylitis?

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OBJECTIVE: Nitric oxide is produced by endothelial nitric oxide synthase, and its production can be influenced by polymorphisms of the endothelial nitric oxide synthase gene. Because candidate genes responsible for susceptibility to ankylosing spondylitis are mostly unknown and available data suggest that there may be problems related to the nitric oxide pathway, such as endothelial dysfunction and increased asymmetric dimethylarginine, this study aimed to assess the association of common endothelial nitric oxide synthase gene polymorphisms with ankylosing spondylitis.

METHODS: One hundred ninety-four unrelated Turkish ankylosing spondylitis patients and 113 healthy without apparent cardiovascular disease, hypertension or diabetes mellitus were included. All individuals were genotyped by PCR-RFLP for two single-nucleotide polymorphisms, namely 786T>C (rs2070744, promoter region) and 786 Glu298Asp (rs1799983, exon 7). Variable numbers of tandem repeat polymorphisms in intron 4 were also studied and investigated by direct electrophoresis on agarose gel following polymerase chain reaction analysis. The Bath ankylosing spondylitis metrology index of the patients was calculated, and human leukocyte antigen B27 was studied.

RESULTS: All studied polymorphisms satisfied Hardy-Weinberg equilibrium. Sex distributions were similar between the patient and control groups. No significant differences were found in the distributions of allele and genotype frequencies of the studied endothelial nitric oxide synthase polymorphisms between patients and controls. There were no correlations between endothelial nitric oxide synthase polymorphisms, disease duration, Bath ankylosing spondylitis metrology index or human leukocyte antigen B27.

CONCLUSION: The results presented in this study do not support a major role of common endothelial nitric oxide synthase polymorphisms in Turkish ankylosing spondylitis patients.

KEYWORDS: Ankylosing Spondylitis; Endothelial Nitric Oxide Synthase; Nitric Oxide; Inflammation; Atherosclerosis.

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disease of the spine and sacroiliac joints. Although the pathogenesis of AS is not known, genetic factors play an important role in determining susceptibility and disease severity. HLA–B27 is the major gene associated with AS. Several non-MHC genes have also been linked to the disease (1). In recent years, there has been considerable interest regarding early atherosclerosis and AS (2-4). In this respect, several studies have suggested an increased prevalence of endothelial dysfunction, an early step in the pathogenesis of atherosclerosis (2,3,5,6), in AS. It is well-known that nitric oxide (NO) is essential in the maintenance of vascular tonus (7) and that the presence of endothelial impairment (reduced vascular relaxation) may suggest a problem regarding the NO pathway. In addition, a considerable number of studies have reported that asymmetric dimethylarginine (ADMA),...
an endogenous NO inhibitor, is significantly increased in the blood of AS patients (8-10). Both endothelial impairment and increased ADMA concentrations suggest the possibility of a problem related to the NO pathway in AS. NO is produced by endothelial NO synthase (eNOS), and its production can be influenced by polymorphisms of the eNOS gene (7). Because candidate genes responsible for susceptibility to AS are mostly unknown and available data suggest the possibility of a problem related with the NO pathway, this study aimed to assess the association of common eNOS gene polymorphisms with AS.

## METHODS

### Sample size, patients and controls

Sample size was calculated using the results of previous studies that investigated eNOS gene polymorphisms in Turkish patients with inflammatory rheumatic diseases based on \( \alpha = 0.05 \) and a power of 80% (11,12). The minimum minor allele frequencies required for this estimation for each group were as follows: Exon 7 (G-894T), 96 subjects; promoter (T-786C), 94 subjects; and intron 4 (variable number of tandem repeat polymorphisms, VNTR), 58 subjects.

We included 194 unrelated AS patients diagnosed according to the modified New York criteria (13). Patients without a history of hypertension (current anti-hypertensive treatment and/or observation of blood pressure levels >140/90 mmHg), diabetes mellitus (participants who reported having ever been told by a physician that they have diabetes mellitus or who reported taking insulin or pills to lower blood glucose levels) or coronary artery disease (history of myocardial infarction, angina pectoris or coronary artery angioplasty) were recruited consecutively from the rheumatology outpatient clinics of the Dokuz Eylül University and three training hospitals (Ataturk, Bozyaka and Tepecik) located in Izmir city. A total of 113 healthy controls subject undergoing the same exclusion criteria as the patients and who did not have any first-degree relatives with diagnoses of AS or related spondyloarthropathies were recruited from the relatives of health professionals and blood donors. Spinal mobility was assessed by the Bath Ankylosing Spondylitis Functional Index (BASFI) (15) and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (16). The Ministry of Health of Turkey Ethics Committee approved the study, and all individuals provided informed consent prior to blood collection in compliance with the principles of the Declaration of Helsinki.

### Genotyping

For all subjects, peripheral blood samples were collected into sterile tubes with EDTA. Genomic DNA was extracted from whole blood using standard proteinase K digestion and the salt-chloroform method. The PCR-RFLP analysis method was used to evaluate the association between three polymorphisms, VNTR), 58 subjects.

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Statistical analysis

MedCalc software, version 12.3.0.0 (MedCalc, Mariakerke, Belgium), was used to estimate sample size. The rest of the statistical analysis was performed on SPSS v. 16.0 software (SPSS Inc, Chicago, IL). Data are expressed as the means ± SDs for continuous variables or as percentages of the total for categorical variables. Pearson χ² or Fisher’s exact tests were used to assess intergroup significance, and a Student’s t test was used to determine differences in means. The distribution of the control genotypes was checked for the Hardy–Weinberg equilibrium. The overall distributions of alleles and genotypes for each polymorphism were compared between cases and controls using χ² analyses. Associations between alleles and genotypes and other variables were examined by χ² tests. An analysis of covariance (ANCOVA) was used to control for confounding variables. A double-tailed p-value of < 0.05 was considered statistically significant.

## RESULTS

There were 194 AS patients (139 male [M], 55 female [F]; 41.5±10.8 years) and 113 healthy controls (69 M, 44 F; 38.6±11.1 years). Sex distributions were similar between the patient and control groups (p = 0.07); however, age was significantly lower in the control group compared with the patients (p = 0.03). Disease duration for the patients was 14.9±9.5 years. HLA-B27 was positive in 72% of patients. The mean BASMI (0-10), BASFI (0-10) and BASDAI (0-10) values were 2.8±2.4, 3.2±2.3 and 3.2±2.5, respectively. The clinical and demographical features of the patient and control groups are given in Table 1.

All studied polymorphisms satisfied the Hardy-Weinberg equilibrium in the controls (eNOS T-786C: X²(1) = 1.96, p = 0.16; eNOS 4b4a VNTR: X²(1) = 2.74, p = 0.09; eNOS Glu298Asp: X²(1) = 1.21, p = 0.27).
Genotype distributions
The frequencies of the T/C polymorphisms at position -786 in the promoter region (patients vs. controls) were as follows: TT (46.3% vs. 34.8%), TC (46.3% vs. 53.6%) and CC (7.4% vs. 11.6%) (p = 0.12). The frequencies of polymorphisms in exon 7 (Glu298Asp; patients vs. controls) were as follows: GG (57.5% vs. 50.9%), GT (35.8% vs. 43.8%) and TT (6.7% vs. 5.4%) (p = 0.36). The frequencies of 4b4a VNTR (intron 4) polymorphisms (patients vs. controls) were as follows: bb (71.7% vs. 65.2%), ba (26.7% vs. 33.9%) and aa (1.6% vs. 9.0%) (p = 0.03). After controlling for age and sex, the distributions of the genotypes (T-786C, Glu298Asp and 4b4a VNTR) were still not significantly different between the patients and controls (p = 0.06, 0.43 and 0.38, respectively).

Allelic distributions
eNOS T-786C (patients vs. controls) was distributed as T (69.4% vs. 61.6%) and C (30.6% vs. 38.4%) (p = 0.07). Glu298Asp (patients vs. controls) was distributed as G (75.4% vs. 72.8%) and T (24.6% vs. 27.2%) (p = 0.5). 4b4a VNTR (patients vs. controls) was distributed as b (85.1% vs. 82.1%) and a (14.9% vs. 17.9%) (p = 0.36). There were still no significant differences between the groups regarding T-786C, Glu298Asp and 4b4a VNTR alleles after controlling for age and sex (p = 0.08, 0.44 and 0.48, respectively). A summary of the allele and genotype frequencies of the three investigated eNOS gene polymorphisms in AS patients and healthy controls is given in Table 2.

Correlation analysis
The correlation analysis showed that eNOS gene polymorphism T-786C was not correlated with the variables of disease duration, BASMI, BASFI, BASDAI or HLAB27 (p = 0.2, 0.1, 0.4, 0.4 and 0.8, respectively). These variables were also not correlated with the Glu298Asp (p = 0.3, 0.3, 0.2, 0.4 and 0.6, respectively) or 4b4a VNTR (p = 0.9, 0.6, 0.4 and 0.2, respectively) polymorphisms.

Table 1 - Clinical and demographic characteristics of the patients and controls.

|                      | AS patients (n = 194) | Healthy controls (n = 113) | p-value |
|----------------------|-----------------------|----------------------------|---------|
| Age (years)          | 41.5 ± 10.8           | 38.6 ± 11.1                | 0.03    |
| Sex, M/F             | 139/55                | 69/44                      | 0.07    |
| HLAB27 positivity, % | 72                    |                            |         |
| Disease duration (years) | 14.9 ± 9.5      |                            |         |
| BASMI (0-10)         | 2.8 ± 2.4             |                            |         |
| BASFI (0-10)         | 3.2 ± 2.3             |                            |         |
| BASDAI (0-10)        | 3.2 ± 2.5             |                            |         |

Continuous data are presented as the means ± standard deviations. BASMI = Bath Ankylosing Spondylitis Metrology Index, BASFI = Bath Ankylosing Spondylitis Functional Index, BASDAI = Bath Ankylosing Spondylitis Disease Activity Index.

Table 2 - Allele and genotype frequencies of the eNOS gene polymorphisms in patients with AS and healthy controls.

| eNOS -786 | Alleles % (n) | Genotypes % (n) |
|-----------|---------------|-----------------|
|           | T             | C               | TT               | T/C | CC   |
| Patients (n = 188) | 69.4 (261)    | 30.6 (115)      | 46.3 (87)        | 46.3 (87) | 7.4 (14) |
| Controls (n = 112) | 61.6 (138)    | 38.4 (86)       | 34.8 (39)        | 53.6 (60) | 11.6 (13) |
| p-value     | 0.07          |                 | 0.12             |     |      |
| eNOS 27 bp VNTR | b             | a               | bb              | ba  | aa  |
| Patients (n = 191) | 85.1 (325)    | 14.9 (57)       | 71.7 (137)       | 26.7 (51) | 1.6 (3) |
| Controls (n = 112) | 82.1 (184)    | 17.9 (40)       | 65.2 (73)        | 33.9 (38) | 0.9 (1) |
| p-value     | 0.36          |                 | 0.3              |     |      |
| eNOS 894 (Glu298Asp) | G             | T               | GG              | GT  | TT  |
| Patients (n = 193) | 75.4 (291)    | 24.6 (95)       | 57.5 (111)       | 35.8 (69) | 6.7 (13) |
| Controls (n = 112) | 72.8 (163)    | 27.2 (61)       | 50.9 (57)        | 43.8 (49) | 5.4 (6) |
| p-value     | 0.5           |                 | 0.36             |     |      |

Note that no significant differences were found in the distributions of allele and genotype frequencies of the studied eNOS gene polymorphisms between patients and controls.
studied. In particular, a single nucleotide polymorphism in the promoter region (T-786C), a 894G>T polymorphism leading to amino acid substitution at position 298 (Glu298Asp, rs1799983) in exon 7, and the 484a polymorphism (a VNTR) located in intron 4 of the eNOS have received the most attention due to their functional relevance to eNOS activity (19). It has been shown that the T-786C polymorphism reduces eNOS gene promoter activity and affects eNOS protein expression, while the Glu298Asp polymorphism causes a structural change in the eNOS protein associated with impaired eNOS activity (20,21). In addition, it has also been shown that the 27bp-VNTR polymorphism reduces the plasma concentration of NO (22). The dysregulation of eNOS caused by these gene polymorphisms leads to decreased NO production and is thought to contribute to the pathogenesis of several diseases, including inflammatory disorders. In this study, we investigated three common eNOS gene polymorphisms (T-786C, Glu298Asp = G894T and 484a VNTR) that have been shown to be associated with eNOS activity. It has been reported that these polymorphisms are associated with CV risk factors and CV disease (7). In the current study, to avoid the confounding effects of these variables on our parameters, we excluded subjects with hypertension, diabetes mellitus and CVD. We also performed statistical adjustments for age and sex to minimize the effects of demographics on our results.

Because of its relationship with inflammation, several studies have investigated the association of eNOS gene polymorphisms with inflammatory rheumatic diseases, including rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and Behcet’s disease (11,12,17,23,24). Some of these studies have yielded evidence of an association of the eNOS polymorphisms with either the pathogenesis of rheumatic diseases or extraarticular manifestations (12,17,23,24). It is well known that there is a strong genetic basis for AS, and some genes, particularly HLAB27, have already been implicated in disease pathogenesis. In recent years, several other non-MHC genes, including genes involved in intracellular antigen processing and cytokine production (especially genes in the IL–17–IL–23 pathway), have also been shown to be related to AS (1). However, it is not known whether AS is associated with eNOS gene polymorphisms.

In the current study, we investigated eNOS gene polymorphisms in AS patients for the following reasons. (1) Previous studies have reported an increased frequency of impaired vascular relaxation (which is known to be mediated by NO) (2-4,6) and higher levels of ADMA (8-10). Although ADMA impairs post-NO production (unrelated to eNOS polymorphisms), it is responsible for the decreased NO activity (8-10). Based on these findings, we suggest that the NO pathway may be impaired and that this might be associated with genetic polymorphisms in the eNOS gene. (2) Some reports have revealed an association between eNOS gene polymorphisms and inflammatory diseases (11,12,17,23,24). Because the pathogenesis of the genes is not fully understood in such inflammatory diseases, we wondered whether there was a link between eNOS gene polymorphisms and AS. We did not find any differences between patients and controls regarding common eNOS gene polymorphisms. We also did not find any relationship between eNOS gene polymorphisms, HLAB27, disease severity, function or activity.

We acknowledge that there were some limitations in our study. (1) We did not study the surrogate markers of atherosclerosis or biomarkers such as carotid intima-media thickness, flow-mediated dilatation and ADMA in this study. Upon consideration, the use of these parameters may be more appropriate for drawing conclusions about eNOS polymorphisms in AS. (2) The inclusion of AS patients with CV risk factors may be more useful for understanding the significance of these polymorphisms in patients with or without CV conditions. In conclusion, despite these limitations, the results presented in this study do not provide support for a major role of common eNOS polymorphisms in Turkish AS patients. Further replication studies in different populations with larger numbers of patients are needed to confirm our results.

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AUTHOR CONTRIBUTIONS

Sari I wrote the manuscript, designed the study and performed the statistical analysis of results. Igar ZY, Gogebakan B and Eslik Z carried out the laboratory analyses. Adam S and Akkor N helped with the general design of the manuscript and contributed to the discussion section. Taylan A, Can G and Bozkaya G collected the patient data. Solmaz D contributed to the statistical analysis and collected the patient data.

REFERENCE

1. Reveille JD. Genetics of spondyloarthritides--beyond the MHC. Nat Rev Rheumatol. 2012;8(5):286-304, http://dx.doi.org/10.1038/nrrheum.2012. 41.
2. Bodnar N, Kerekes G, Seres I, Paragh G, Kappelmayer J, Nemethne ZG, et al. Assessment of subclinical vascular disease associated with ankylosing spondylitis. J Rheumatol. 2011;38(4):723-9, http://dx.doi. org/10.3899/jrheum.100668.
3. Gonzalez-Juanatey C, Vazquez-Rodriguez TR, Miranda-Filloy JA, Dierssen T, Vaqueiro I, Blanco R, et al. The high prevalence of subclinical atherosclerosis in patients with ankylosing spondylitis without clinically evident cardiovascular disease. Medicine (Baltimore). 2009;88(6):358-65.
4. Sari I, Okan T, Akar S, Cece H, Altay C, Secil M, et al. Impaired endothelial function in patients with ankylosing spondylitis. Rheumatol (Oxford). 2006;45(3):283-6.
5. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993;362(6423):801-9, http://dx.doi.org/10.1038/362801a0.
6. Azevedo VF, Pecoits-Filho R. Atherosclerosis and endothelial dysfunction in patients with ankylosing spondylitis. Intern Med. 2009;48(16):1363-8, http://dx.doi.org/10.1007/s11586-009-0097-4.
7. Cooke GE, Doshi A, Binkley PF. Endothelial nitric oxide synthase gene: prospects for treatment of heart disease. Pharmacogenomics. 2007;8(12):1723-34, http://dx.doi.org/10.2217/14622416.8.12.1723.
8. Heniy-Beka E, Gesztesy R, Bodnar N, Zeuga J, Kerekes G, Zeuga M, et al. Increased production of asymmetric dimethylarginine (ADMA) in ankylosing spondylitis: association with other clinical and laboratory parameters. Joint Bone Spine. 2011;78(2):184-7, http://dx.doi.org/10.1016/j.jbspin.2010.05.009.
9. Sari I, Kebapcular L, Alacacioglu A, Bilgir O, Yildiz Y, Taylan A, et al. Increased levels of asymmetric dimethylarginine (ADMA) in patients with ankylosing spondylitis. Intern Med. 2009;48(10):1363-8, http://dx.doi.org/10.2217/14622416.8.12.1723.
10. Erre GL, Sanna P, Zinellu A, Ponchietti A, Fenu P, Setgia S, et al. Plasma asymmetric dimethylarginine (ADMA) levels and atherosclerotic disease in ankylosing spondylitis: a cross-sectional study. Clin Rheumatol. 2010;29(10):1487-93, http://dx.doi.org/10.1007/s12067-010-1388-5.
11. Olsel F, Keser G, Ozmen M, Aksu K, Kitapcioglu G, Berdili A, et al. Endothelial nitric oxide synthase gene Glu298Asp polymorphism is associated with Behcet’s disease. Clin Exp Rheumatol. 2006;24(5 Suppl 42):S79-82.
12. Sinici I, Kalyoncu U, Karahan S, Kiraz S, Atalar E. Endothelial nitric oxide gene polymorphism and risk of systemic sclerosis: predisposition effect of T-786C promoter and protective effect of 27 bp repeats in Intron 4. Clin Exp Rheumatol. 2010;28(2):169-75.
13. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum. 1984;27(4):361-8, http://dx.doi.org/10.1002/art.1780270401.

14. Jenkinson TR, Mallorie PA, Whitelock HC, Kennedy LG, Garrett SL, Calin A. Defining spinal mobility in ankylosing spondylitis (AS). The Bath AS Metrology Index. J Rheumatol. 1994;21(9):1694-8.

15. Calin A, Garrett S, Whitelock H, Kennedy LG, O’Hea J, Mallorie P, et al. A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index. J Rheumatol. 1994;21(12):2281-5.

16. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol. 1994;21(12):2286-91.

17. Nagy G, Koncz A, Telarico T, Fernandez D, Ersek B, Buzas E, et al. Central role of nitric oxide in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. Arthritis Res Ther. 2010;12(3):210, http://dx.doi.org/10.1186/ar3045.

18. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33(7):829-37, 837a-837d, http://dx.doi.org/10.1093/eurheartj/ehr304.

19. Thameem F, Puppala S, Arar NH, Stern MP, Blangero J, Duggirala R, et al. Endothelial nitric oxide synthase (eNOS) gene polymorphisms and their association with type 2 diabetes-related traits in Mexican Americans. Diab Vasc Dis Res. 2008;5(2):109-13, http://dx.doi.org/10.3132/dvdr.2008.018.

20. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, et al. T-786-->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation. 1999;99(22):2864-70, http://dx.doi.org/10.1161/01.CIR.99.22.2864.

21. Yoshimura M, Yasue H, Nakayama M, Shimasaki Y, Sumida H, Sugiyama S, et al. A missense Gln298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. Hum Genet. 1998;103(1):65-9, http://dx.doi.org/10.1007/s004390050785.

22. Tsukada T, Yokoyama K, Arai T, Takemoto F, Haras S, Yamada A, et al. Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans. Biochim Biophys Res Commun. 1998;245(1):190-3, http://dx.doi.org/10.1006/bbrc.1998.8267.

23. Lee YH, Lee HS, Choi SJ, Ji JD, Song GG. Associations between eNOS polymorphisms and susceptibility to systemic lupus erythematosus: a meta-analysis. Inflamm Res. 2012;61(2):135-41, http://dx.doi.org/10.1007/s00011-011-0397-3.

24. Melchers I, Blaschke S, Hecker M, Cattaruzza M. The -786C/T single-nucleotide polymorphism in the promoter of the gene for endothelial nitric oxide synthase: insensitivity to physiologic stimuli as a risk factor for rheumatoid arthritis. Arthritis and rheumatism. 2006;54(10):3144-51, http://dx.doi.org/10.1002/art.22147.