



## Lack of association of the *G22A* polymorphism of the *ADA* gene in patients with ankylosing spondylitis

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**ABSTRACT.** Genes located outside the HLA region (6p21) have been considered as candidates for susceptibility to ankylosing spondylitis. We tested the hypothesis that the *G22A* polymorphism of the adenosine deaminase gene (*ADA*; 20q13.11) is associated with ankylosing spondylitis in 166 Brazilian subjects genotyped for the *HLA*\*27 gene (47 patients and 119 controls matched for gender, age and geographic origin). The *HLA-B*\*27 gene and the *G22A ADA* polymorphism were identified

by PCR with sequence-specific oligonucleotide probes and PCR-RFLP, respectively. There were no significant differences in frequencies of *ADA* genotypes [odds ratio (OR) = 1.200, 95% confidence interval (CI) = 0.3102-4.643,  $P > 0.8$ ] and *ADA\*01* and *ADA\*02* alleles (OR = 1.192, 95%CI = 0.3155-4.505,  $P > 0.8$ ) in patients versus controls. We conclude that the *G22A* polymorphism is not associated with ankylosing spondylitis.

**Key words:** Ankylosing spondylitis; Adenosine deaminase polymorphism; HLA-B\*27; SSOP

## INTRODUCTION

Ankylosing spondylitis is an autoimmune disease characterized by inflammation in the spine. The disease process can result in stiffness and functional limitations of the axial skeleton (Van der Linden and Van der Heijde, 1998). This disease mainly affects white men during the second and fourth decades of life and is strongly associated with human leukocyte antigen (HLA) gene *HLA-B\*27*. However, patients without this gene are also affected (Lopes de Castro, 2007), which suggests that additional genes not linked to the HLA system (6p21) influence the susceptibility and possibly the severity to this disease (Brionez and Reveille, 2008).

The polymorphic enzyme adenosine deaminase (ADA) is encoded by the gene *ADA* (20q13.11) and regulates intracellular and extracellular adenosine concentrations. The *G22A* polymorphism of *ADA*, in addition to defining the alleles *ADA\*01* (*G*) and *ADA\*02* (*A*), is the only known polymorphism altering the expression of ADA levels (Hirschhorn et al., 1994).

Polymorphisms of the *ADA* gene influence immune reactions (Bottini et al., 1981) and susceptibility to autoimmune diseases (Sasaki, 1984). Some independent studies have reported an association between the *G22A* polymorphism and rheumatoid arthritis (Sebastiani et al., 2010) and type 1 diabetes (Saccucci et al., 2009, 2010), whereas others have found a relationship between the plasma ADA concentration and clinical variability in rheumatoid arthritis (Sari et al., 2003; Wessles et al., 2006). The aim of this study was to test the hypothesis that the *G22A* polymorphism of *ADA* is associated with ankylosing spondylitis.

## MATERIAL AND METHODS

### Ethical considerations

This study was approved by the Research Ethics Committee of the Medical School in São José do Rio Preto (055/2011). Each participant signed a consent form after receiving information about the objectives and procedures of the study.

### Selection of participants

One hundred and fifty-six patients suspected of having spondyloarthropathy were referred to the Molecular Immunogenetics Laboratory of the Blood Bank in São José do Rio Preto for an investigation of *HLA-B\*27*. Of these patients, 47 who were diagnosed with ankylosing spondylitis confirmed by the Rheumatology Outpatient Clinic of FUNFARME according to mod-

ified criteria of New York (Goie et al., 1985) and the Brazilian Consensus for the Diagnosis and Treatment of Spondyloarthropathies (Sampaio-Barros et al., 2007) were selected for this study. The main clinical signs obtained from the medical records were axial inflammatory pain (N = 47), bilateral sacroiliitis with grades ranging from 1 to 4 (N = 47), and asymmetric synovitis (N = 13).

A second group was composed of 119 healthy individuals who spontaneously went to the same blood bank and were considered eligible for blood donation and registered as volunteer bone marrow donors. According to Brazilian guidelines, all blood donors are subjected to screening tests for 20 infectious diseases ([http://portal.saude.gov.br/portal/arquivos/pdf/resolucao\\_153\\_2004.pdf](http://portal.saude.gov.br/portal/arquivos/pdf/resolucao_153_2004.pdf)). Each donor was interviewed to obtain data on cases of autoimmune diseases in their families. Patients and controls were matched by age, gender, and geographic origin.

### **Blood collection and genomic DNA extraction**

A 5-mL sample of peripheral blood was drawn from each individual via venipuncture into vacuum tubes with ethylenediaminetetraacetic acid for genomic DNA extraction using a commercial kit (Purelink, Invitrogen).

### **Identification of *HLA-B\*27***

*HLA-B\*27* was identified using a Dynal HLA-B Reli™ SSO Typing Kit (Invitrogen) following manufacturer recommendations.

### **Identification of the *G22A* polymorphism and *ADA\*01* and *ADA\*02***

To characterize the *G22A* polymorphism of *ADA* and identify *ADA\*01* and *ADA\*02*, we used a published polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol (Safranow et al., 2007). Briefly, an amplified fragment of the *ADA* gene with primers (sense: 5'-GCCCGGCCCGTTAAGAAGAGC-3'; anti-sense: 5'-GGTCAAGTCAGGGGCAGAAGCAGA-3') containing 397 bp was digested using *TaqI* restriction enzyme, generating 2 fragments for *ADA\*1* (152 and 245 bp) or an undigested fragment (397 bp) for *ADA\*2*. All procedures were analyzed using 2% agarose gel electrophoresis in TEB buffer.

### **Statistical analysis**

Data from patients and controls were compared with Fisher exact, chi-square, and *t*-tests using GraphPad InStat version 3.06. The odds ratio (OR) and 95% confidence interval (95%CI) were also calculated. A P value of  $\leq 0.05$  was considered significant. The Online Encyclopedia for Genetic Epidemiology Studies ([www.oege.org/software/hwe-mr-calc.shtml](http://www.oege.org/software/hwe-mr-calc.shtml)) was used to assess the distribution of *ADA* genotypes according to Hardy-Weinberg equilibrium.

## **RESULTS**

Table 1 shows the characteristics of patients and controls and the frequencies of *ADA* genotypes and alleles. No statistically significant differences were identified in a comparison

of the mean ages of the two groups. The incidence of ankylosing spondylitis was 2.6 times higher in men than in women. The frequencies of the ADA genotypes and alleles did not differ significantly between patients and controls (genotypes:  $P = 1.000$ , OR = 1.200, 95%CI = 0.3102-4.643; alleles:  $P = 1.000$ , OR = 1.192, 95%CI = 0.3155-4.505) and were in agreement with Hardy-Weinberg equilibrium (patients:  $\chi^2 = 0.05$ , d.f. = 1; controls =  $\chi^2 = 0.18$ , d.f. = 1). For the patients, the mean age at onset of symptoms was  $37.7 \pm 10.7$  years, ranging from 16 to 79 years. The differences in the frequencies of HLA-B\*27 between patients and controls, as expected, were statistically significant ( $P < 0.0001$ ; OR = 30.343; 95%CI = 11.048-83.330).

**Table 1.** Gender, mean age and ADA genotypes and alleles of patients and controls.

	Patients (N = 47)		Controls (N = 119)				P*
	N	%	N	%	OR	95%CI	P
Gender (M/F)	34/13		83/36				0.850
Mean age (SD)	50.3 ( $\pm 10.5$ )		49.5 ( $\pm 10.5$ )				0.658**
Genotypes							
*01;*01	44	93.6	110	92.4	1.200	0.3102-4.643	1.000
*01;*02	3	6.4	9	7.6			
Alleles							
*01	91	96.8	229	96.2	1.192	0.3155-4.505	1.000
*02	3	3.2	9	3.8			

\*Calculated by the exact Fisher test. \*\*Calculated by the *t*-test.

Table 2 shows the frequencies of ADA\*01;\*01 and ADA\*01;\*02 genotypes according to the presence and absence of HLA-B\*27. The differences in the frequencies were not statistically significantly for patients and controls, both HLA-B\*27 positive ( $P = 0.441$ ; OR = 2.700; 95%CI = 0.203-35.768) and HLA-B\*27 negative ( $P = 1.000$ ; OR = 1.295; 95%CI = 0.152-11.026).

**Table 2.** ADA genotype frequencies according to the presence and absence of the HLA-B\*27 gene in patients and controls.

Genotypes	Patients (N = 47)				Controls (N = 119)			
	HLA-B*27 (+)		HLA-B*27 (-)		HLA-B*27 (+)		HLA-B*27 (-)	
	N	%	N	%	N	%	N	%
ADA*01;*01	27	93.1	17	94.4	5	83.3	105	92.9
ADA*01;*02	2	6.9	1	5.6	1	16.7	8	7.8
Total	29	61.7	18	38.3	6	5.0	113	95.0

(+) = HLA-B\*27 present; (-) = HLA-B\*27 absent. Patients versus controls, both HLA-B\*27 (+):  $P = 0.441$ , OR = 2.700, 95%CI = 0.203-35.768. Patients versus controls, both HLA-B\*27 (-):  $P = 1.000$ , OR = 1.295, 95%CI = 0.152-11.026.

The distribution of the main clinical signs (axial inflammatory pain, bilateral sacroiliitis with degrees ranging from 1 to 4, and asymmetric synovitis) showed no statistically significant differences between patients with ADA\*01;\*01 and ADA\*01;\*02 genotypes independent of the presence or absence of HLA-B\*27.

## DISCUSSION

Genetic factors play an important role in the genesis of autoimmunity (Lopes de Castro, 2007) and growing evidence shows that genes located outside the HLA region influence suscep-

tibility to ankylosing spondylitis (Brionez and Reveille, 2008). To test the hypothesis that that *G22A* polymorphism of *ADA* is associated with ankylosing spondylitis, patients with ankylosing spondylitis and a control group of healthy individuals matched for age, gender, and geographic origin were enrolled to compare the results. To the best of our knowledge, this study is the first to evaluate the *G22A* polymorphism of *ADA* in Brazilian patients with ankylosing spondylitis.

The medical literature suggests that the prevalence and incidence of ankylosing spondylitis is higher in white men carrying the gene *HLA-B\*27* and that symptoms of the disease begin appearing between the second and fourth decades of life (Van der Linden and Van der Heijde, 1998; Breban, 2006; Lopez de Castro, 2007). The results of this study corroborate these statements. In fact, the cases selected for this study present some degree of mixing, but it seems that the miscegenation did not eliminate the differential impact of this disease on gender, age group of highest incidence, or presence of its main genetic marker.

The *ADA* genotype frequencies complied with the Hardy-Weinberg equilibrium in patients and controls. Although ankylosing spondylitis is a complex disease influenced by genetic, environmental, and immune factors (Van der Linden and Van der Heijde, 1998; Lopez de Castro, 2007; Brionez and Reveille, 2008), the results reported here showed no deviations from the expected genotype proportions for patients and controls. Only the *ADA\*01; \*01* and *ADA\*01; \*02* genotypes were found in patients and controls, but the differences between their frequencies were not statistically significant. These data disagree with reports of associations between *G22A ADA* polymorphism and rheumatoid arthritis (Sebastiani et al., 2010) and type 1 diabetes (Saccucci et al., 2009, 2010). Various factors could contribute for the disagreement. Previous studies (Saccucci et al., 2009, 2010; Sebastiani et al., 2010) were carried out in white Europeans, and the patients enrolled in this study introduced African influence (SEADE, 2009). This distinct genetic background could account for the differences reported herein.

It has been reported that the frequency of *ADA\*02* is four times lower in African descendants than in whites (Hirschhorn et al., 1994). Two independent Brazilian studies have confirmed these data. Dutra et al. (2010) reported a frequency for this allele that is two times higher in the Rio Grande do Sul State than that in the northwestern region of São Paulo State (Nunes et al., 2011). In fact the ethnic background of the population living in these states is different, with a predominance of whites in the Brazilian southern region (Dutra et al., 2010). It seems that the frequency of the *ADA\*2* allele among Brazilians is influenced by ethnic and racial admixture.

The frequencies of *ADA\*01; \*01* and *ADA\*01; \*02* genotypes were compared with the main clinical signs of ankylosing spondylitis (axial pain, synovitis of the lower limbs, and grades 2 to 4 sacroiliitis), but the differences were not significant. In the light of this study, we can assume that the *G22A ADA* polymorphism is not associated with the variability of clinical symptoms in ankylosing spondylitis. This finding is in accordance with a previous Brazilian report, which demonstrated no association between the serum ADA concentration and the clinical variability of systemic lupus erythematosus (Lima et al., 2005).

Evidence suggests that *ADA\*02* exerts a protective effect in inflammatory diseases such as asthma (Liu et al., 2006) and coronary artery disease (Safranow et al., 2007). Additionally, the haplotype 2<sup>2</sup>/6<sup>1</sup> of *ADA*, which contains *ADA\*02*, seems to exert protective action against type 1 diabetes in men (Saccucci et al., 2010). The influence of the plasma ADA concentration and clinical variability in rheumatoid arthritis has also been reported (Sari et al., 2003; Wessels et al., 2006). These data lead to the assumption that the *G22A* polymorphism

has some biological relationship with inflammatory and autoimmune diseases.

This study failed to demonstrate any association between the *G22A ADA* polymorphism and ankylosing spondylitis. The disagreement between the results of this and other studies may stem from several factors. This polymorphism may exert a small influence in ankylosing spondylitis, but its contribution may be obscured by the strong association of this disease with *HLA-B\*27*. We compared the frequencies of *ADA\*01* and *ADA\*02* with the presence and absence of *HLA-B\*27* in patients and controls. The frequency of the *ADA\*01; \*02* genotype was 2 times higher in *HLA-B\*27*-positive controls than in patients with ankylosing spondylitis, but the difference was not statistically significant. Apparently, preferential association of *ADA* genotypes and *HLA-B\*27* occurs in both patients and controls. However, owing to the small number of patients with ankylosing spondylitis enrolled in this study, the results should be considered a preliminary assessment that must be confirmed in other populations.

Further investigations are needed to clarify whether variations in the concentration of the adenosine deaminase enzyme resulting from the *G22A* polymorphism influence susceptibility, clinical variability, and therapeutic response in ankylosing spondylitis. Our results indicate that the *G22A* polymorphism of *ADA* in isolation or together with *HLA-B\*27* is not associated with ankylosing spondylitis.

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