SOCIEDADE BRASILEIRA DE REUMATOLOGIA

REVISTA BRASILEIRA DE REUMATOLOGIA

www.reumatologia.com.br

Original article

Clinical characteristics and frequency of TLR4 polymorphisms in Brazilian patients with ankylosing spondylitis

Natalia Pereira Machado[a], *Eliana Nogueira*[b], *Karen Oseki*[a], *Pâmela Carolina Cruz Ebbing*[a], *Clarice Silvia Taemi Origassa*[b], *Tatiane Mohovic*[b], *Niels Olsen Saraiva Câmara*[b], *Marcelo de Medeiros Pinheiro*[a,*]

[a] *Divisão de Reumatologia, Departamento de Medicina, Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil*
[b] *Divisão de Nefrologia, Departamento de Medicina, Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 10 February 2016
Accepted 1 May 2016
Available online 5 August 2016

Keywords:
Ankylosing spondylitis
TLR-4 polymorphisms
HLA-B27

ABSTRACT

Objectives: Innate immunity is involved in the physiopathology of ankylosing spondylitis (AS), with the participation of Gram-negative bacteria, modulation of human leukocyte antigen (HLA) B27 and the involvement of pattern recognition receptors, such as Toll-like receptors (TLRs). The aim of this study was to investigate the clinical characteristics and frequency of TLR4 polymorphisms (Asp299Gly and Thr 399Ile) in a cohort of Brazilian patients with AS.

Methods: A cross-sectional study was carried out involving 200 patients with a diagnosis of AS and a healthy control group of 200 individuals. Disease activity, severity and functional capacity were measured. The study of TLR4 polymorphisms was performed using the restriction fragment length polymorphism method. HLA-B27 was analyzed by conventional polymerase chain reaction. The IBM SPSS Statistics 20 program was used for the statistical analysis, with p-values less than 0.05 considered significant.

Results: Mean age and disease duration were 43.1 ± 12.7 and 16.6 ± 9.2 years, respectively. The sample was predominantly male (71%) and non-Caucasian (52%). A total of 66% of the group of patients were positive for HLA-B27. The sample of patients was characterized by moderate functional impairment and a high degree of disease activity. No significant association was found between the two TLR4 polymorphisms and susceptibility to AS.

Conclusions: TLR4 polymorphisms 399 and 299 were not more frequent in patients with AS in comparison to the health controls and none of the clinical variables were associated with these polymorphisms.

* *Corresponding author.*
E-mail: mpinheiro@uol.com.br (M.M. Pinheiro).

http://dx.doi.org/10.1016/j.rbre.2016.07.004

Características clínicas e frequência de polimorfismos em TLR4 em pacientes brasileiros com espondilite anquilosante

Palavras-chave:
Espondilite anquilosante
Polimorfismos em TLR-4
HLA-B27

RESUMO

Objetivos: A imunidade inata está envolvida na fisiopatologia da espondilite anquilosante (EA), com a participação de bactérias gram-negativas, modulação do antígeno leucocitário humano (HLA) B27 e o envolvimento de receptores de reconhecimento de padrões, como os receptores Toll-like (TLR). O objetivo deste estudo foi investigar as características clínicas e a frequência de polimorfismos em TLR4 (Asp299Gly e Thr399Ile) em uma coorte de pacientes brasileiros com EA.

Métodos: Fez-se um estudo transversal que envolveu 200 pacientes com diagnóstico de EA e um grupo controle saudável de 200 indivíduos. Mediram-se a atividade da doença, a gravidade e a capacidade funcional. O estudo dos polimorfismos em TLR4 foi feito com o método de polimorfismo de fragmentos de restrição. O HLA-B27 foi analisado por reação em cadeia da polimerase convencional. Usou-se o programa SPSS Statistics 20 da IBM para a análise estatística e foram considerados significativos valores de p inferiores a 0,05.

Resultados: A média de idade e a duração da doença foram de 43,1 ± 12,7 e 16,6 ± 9,2 anos, respectivamente. A amostra foi predominantemente do sexo masculino (71%) e de não brancos (52%). Do grupo de pacientes 66% eram HLA-B27 positivos. A amostra de pacientes foi caracterizada por uma alteração funcional moderada e um elevado grau de atividade da doença. Não foi encontrada associação estatisticamente significativa entre os polimorfismos em TLR4 e a susceptibilidade à EA.

Conclusões: Os polimorfismos em TLR4 399 e 299 não foram mais frequentes em pacientes com EA em comparação com controles saudáveis e nenhuma das variáveis clínicas esteve associada a esses polimorfismos.

Introduction

Pattern-recognition receptors (PRRs) are a set of receptors involved in recognition of pathogens in multicellular organisms. Toll-like receptors (TLRs)[1] function as PRRs and play an essential role in the recognition of microbial components and endogenous ligands induced during the inflammatory response.[2–4]

Among the gene polymorphisms of TLR, some of the most widely studied are two co-segregated functional mutations in the extracellular domain of human TLR4, which are located on chromosome 4 and are associated with hyporesponsiveness to bacterial lipopolysaccharides (LPS).[5] Based on its evidence of association with an increased risk of infection by Gram-negative bacteria,[5] such polymorphisms have been evaluated in some inflammatory diseases in which the participation of these microorganisms has been implicated in the etiopathology, such as ankylosing spondylitis (AS).

Based on the premise of subclinical colitis in patients with AS and animal models that demonstrate the participation of an infectious trigger by Gram-negative bacilli[6,7] modulated by the presentation of the antigen to HLAB27,[8] the aim of the present study was to identify the frequency of TLR4 polymorphisms (Asp299Gly and Thr399IIe) in Brazilian patients with AS and investigate possible associations between these polymorphisms and greater susceptibility to the disease as well as clinical and laboratory aspects of disease activity and chronicity.

The results of the association between TLR polymorphisms and AS have been controversial in some clinical trials,[9] probably because of different population studied. This fact motivates investigation of this association in miscigenated populations, like Brazilian one.

Materials and methods

Two hundred patients with a diagnosis of AS according to the modified New York criteria[10] or axial spondyloarthritis[11] were recruited from the spondyloarthritis clinic of the Rheumatology Sector of the Federal University of Sao Paulo (Brazil) and 200 healthy individuals were selected from among volunteers giving blood at the hospital of the same institution between May 2011 and October 2013. All participants agreed to participate in the study by signing a statement of informed consent (ethics committee number 1804/10).

Demographic and clinical data were collected and specific assessment tools were employed for the characterization of disease activity and severity, such as the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI),[12] Ankylosing Spondylitis Disease Activity Score (ASDAS),[13] Bath Ankylosing Spondylitis Functional Index (BASFI),[14] Bath Ankylosing Spondylitis Metrology Index (BASMI)[15] and modified Stoke Ankylosing Spondylitis Spine Score (mSASSS).[16]

The analysis of Asp299Gly and Thr399Ile polymorphisms of TLR4 was performed using polymerase chain reaction (PCR) followed by restriction enzyme digestion for each allele

(NCOI for 299 and HinfI for 399).[17,18] GenomicDNA extraction was performed using whole blood collected in ethylenediaminetetraacetate (EDTA) using a DNA extraction kit (DNA NucleoSpin®, Macherey-Nagel). The PCR amplification was performed with 50 ng of the DNA to be studied in a total volume of 20 μL containing 0.8 μL of potassium chloride 50 mM, 2 μL of Tris (pH 8.4), 0.6 mM of magnesium chloride, 0.4 μL of each primer (10 nM), 0.4 μL of deoxyribonucleotide mixture (dATP, dCTP, dGTP and dTTP) and 0.06 μL units of Taq platinum DNA polymerase (0.015 U/μL).

The automatic thermal cycler (MJ Research PTC-200) was programmed for amplification: initial denaturation (95 °C for 4 min), followed by 35 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min 30 s, with final extension at 72 °C for 10 min. The forward and reverse primers were respectively 5′-GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG-3′ and 5′-GAT CAA CTT CTG AAA AAG CAT TCC CAC-3′ for Asp299Gly and 5′-GGT TGC TGT TCT CAA AGT GAT TTT GGG AGA A and 5′-CCT GAA GAC TGG AGA GTG AGT TAA ATG CT-3′ for Thr399Ile. Electrophoresis in 2% agarose gel was performed to confirm the DNA amplification.

An aliquot of 5 μL with the appropriate restriction enzyme was used for digestion of the PCR product at 37 °C for 2 h. Electrophoresis was performed in 4% agarose gel (Agarose 1000 Invitrogen, Eugene, OR, USA) for identification of the TLR4 alleles. The gel was stained with Sybr Gold (Nucleic acid gel stain, Invitrogen, Eugene, OR, USA) and visualized using the Storm 849 system (Molecular Dynamics, USA).

Table 1 – Clinical and demographic data of patients with ankylosing spondylitis and healthy controls.

Clinical characteristics	Patients (*n* = 200)	Controls (*n* = 200)	*p*
Age (years)	43.1 ± 12.7	38.5 ± 11.2	0.001
Male gender (n, %)	142 (71%)	121 (60.5%)	0.027
Caucasian ethnicity (n, %)	96 (48%)		
Symptom duration (years)	16.6 ± 9.2		
Time since diagnosis (years)	7.6 ± 6.2		
Family history of AS (n, %)	46 (23%)		
Presence of HLA-B27 (n, %)	130 (66%)		
Peripheral involvement (n, %)			
Arthritis (past or current)	99 (49.5%)		
Enthesitis (past or current)	129 (64.5%)		
Isolated axial involvement (n, %)	42 (21%)		
Hip involvement (n, %)	26 (13%)		
Anterior uveitis (past or current) (n, %)	74 (37%)		
Current living habits (n, %)			
Smoking	19 (9.5%)		
Regular physical activity	44 (22%)		
Specific disease indices			
BASDAI	2.25 ± 2.02		
BASMI	4.28 ± 2.30		
BASFI	3.80 ± 2.52		
ASDAS-Sed. rate	2.20 ± 1.06		
ASDAS-CRP	2.07 ± 1.08		
HAQ-S	1.05 ± 1.35		
mSSASS	19.0 ± 22.32		
Tests for inflammatory activity			
Sed. rate (mm)	21.92 ± 20.47		
CRP-us (mg/dL)	8.96 ± 13.53		
Current conventional treatment (n, %)			
NSAIDs	125 (62.5%)		
Glucocorticoids	12 (6%)		
Methotrexate	34 (17%)		
Sulfasalazine	18 (9%)		
TNFα inhibitors (n, %)	81 (40.5%)		
Infliximab	35 (17.5%)		
Etanercept	24 (12%)		
Adalimumab	22 (11%)		

AS, ankylosing spondylitis; NSAID, non-steroidal anti-inflammatory drug; BASDAI, Bath ankylosing spondylitis disease activity index; BASMI, Bath ankylosing spondylitis metrology index; BASFI, Bath ankylosing spondylitis function index; ASDAS, Ankylosing spondylitis disease activity score; HAQ-S, Health assessment questionnaire-Spondylitis; mSSASS, modified Stokes ankylosing spondylitis spine score; Sed. rate, blood sedimentation rate; CRP-us, ultrasensitive C-reactive protein.
Mann–Whitney test.

The analysis of HLA-B27 was performed using conventional PCR in an automatic thermal cycler (MJ Research PTC-200): 70 ng of DNA from each sample, 0.9 μmol/L of each forward and reverse primer, 1.1 mmol/L of magnesium chloride, 200 μmol/L of deoxyribonucleotide mixture (dATP, dCTP, dGTP and dTTP) and 2 U of Taq DNA polymerase for a total volume of 25 μL. The forward and reverse primers for the reaction were respectively E91S (5′-GGG TCT CAC ACC CTC CAG AAT-3′) and 136AS (5′-CGG CGG TCC AGG AGC T-3′). The cycling conditions were 100 s at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 64 °C and 2 min at 72 °C, with final extension at 72 °C for 10 min in 40 consecutive cycles.

To evaluate the reaction quality (internal control), reactions were performed using primers for β-globin for all samples under the same conditions used for the HLA-B27 reactions. The primers for these reactions were PCO4 (5′-CAA CTT CAT CCA CGT TCA CC-3′) and GH20 (5′-GAA GAG CCA AGG ACA GGT AC-3′). The PCR products were analyzed through electrophoresis in 1% agarose gel run for one hour at 100 V.

Numerical data were expressed as mean and standard deviation. The Kolmogorov–Smirnov test was used to determine the distribution of the data (normal or non-normal). Either the Mann–Whitney test or Kruskal–Wallis test was used for comparisons among the categorical and numerical data. The chi-square test was used to determine the distribution of the TLR4 polymorphisms between groups as well as for the comparison of the categorical variables. Spearman's correlation coefficients were calculated to determine the strength of correlations among the continuous variables. Bivariate and multivariate logistic regression models were constructed with variables that exhibited significant associations in the previous tests. The IBM SPSS 20 program was used for the statistical analysis, with *p*-values less than 0.05 considered significant.

Results

Table 1 displays the clinical and demographic data of the 200 patients. As expected, the male gender was predominant. Approximately one quarter of the patients had a family history of AS and half the sample reported current or past peripheral involvement. Nearly 40% of the patients had anterior uveitis. The prevalence rates of current smoking and the regular practice of physical exercise were low.

Mean disease activity was high, with significant functional and mobility impairment reflecting the long duration of the disease. More than 60% of the patients made regular use of a non-steroidal anti-inflammatory drug and 35% used a synthetic disease-modifying anti-rheumatic drug. Nearly half the patients used TNFα inhibitors, with equal distribution among etanercept, infliximab and adalimumab.

The study of HLA-B27 was performed on 197 (98.5%) patients with AS and 60 (30%) healthy individuals, 66% ($n = 130$) and 1.6% ($n = 1$) of whom tested positive, respectively. Extra-articular manifestations were found in 80 patients (40%), the most frequent of which was acute anterior uveitis ($n = 74$; 37%), followed by circinate balanitis ($n = 3$; 1.5%), nonspecific colitis ($n = 2$; 1%) and sterile urethritis ($n = 1$; 0.5%).

Table 2 – Frequency of TLR4 polymorphisms (299 and 399) in patients and controls.

TLR-4 polymorphisms	Patients $n = 200$	Controls $n = 200$	*p*
Asp299Gly			
Wild	182 (91%)	178 (89%)	0.505
Heterozygote	17 (8.5%)	22 (11%)	
Homozygote	1 (0.5%)	0	
Thr399Ile			
Wild	187 (93.5%)	186 (93%)	0.50
Heterozygote	13 (6.5%)	14 (7%)	
Homozygote	0	0	

Chi-square test.

No statistically significant differences between the patients and healthy controls were found regarding Asp299Gly and Thr399Ile polymorphisms. Due to the very low number of homozygotes, heterozygous and homozygous patients were included in the same group for the tests. The 299 and 399 polymorphisms were in Hardy–Weinberg (HW) equilibrium in the patients (HW-$\chi^2 = 0.73$, $p = 0.39$ and HW-$\chi^2 = 0.22$, $p = 0.63$, respectively) and controls (HW-$\chi^2 = 0.68$, $p = 0.41$ and HW-$\chi^2 = 0.26$, $p = 0.61$, respectively), demonstrating the conservation of genotype frequencies across generations (Table 2). A tendency was found toward a greater frequency of non-co-segregation of the alleles in the control group in comparison to the patients with AS (Table 3).

For a more detailed analysis, the patients were separated into subgroups based on clinical characteristics. Female patients had a higher body mass index (BMI) as well as higher HAQ-S and ASDAS-Sed. rate scores. Females also had shorter disease duration, lesser severity of sacroiliac involvement as well as lower mSASSS and BASMI scores in comparison to males (Table 3). After controlling for confounding variables in the logistic regression model, only BMI ($p = 0.014$) and the BASMI score ($p = 0.02$) remained significantly associated with gender.

In the logistic regression analysis using positivity for B27 as the dependent variable, significant associations were found for peripheral arthritis ($p = 0.039$), uveitis ($p = 0.033$) and the use of TNFα inhibitors ($p = 0.003$) (Table 4). Among the patients positive for HLA-B27, a tendency was found toward a predominance of Caucasians ($p = 0.058$). Moreover, greater prevalence rates were found in this group regarding anterior uveitis, more severe sacroiliac involvement (Grade IV), longer disease duration and a greater frequency of biological agents ($p < 0.05$) (Table 5).

Patients with the adult form of the disease had higher scores on the BASFI, BASDAI and HAQ-S, were older and had longer disease duration. Those with the juvenile form of the disease more frequent reports of a family history of AS, made more use of sulfasalazine and reported more side effects from TNFα inhibitors. However, none of these variables remained statistically significant in the final multiple regression model.

Non-Caucasian patients had a higher BMI and BASMI score in comparison to Caucasian patients (Table 6). However, only the BASMI score remained significant in the logistic regression

Table 3 – Characteristics of patients with ankylosing spondylities according to gender.

Variables	Male (n = 142)	Female (n = 58)	p
Ethnicity			
Caucasian	67 (47.2%)	29 (50%)	
Non-Caucasian	75 (52.8%)	29 (50%)	0.717
Onset			
Adult	118 (83.1%)	50 (86.2%)	
Juvenile	24 (16.9%)	8 (13.8%)	0.586
HLA-B27+	96 (67.6%)	34 (58.6%)	0.158
Arthritis	68 (47.9%)	31 (53.4%)	0.475
Entesitis	93 (65.5%)	36 (62%)	0.646
Anterior uveitis	53 (37.3%)	21 (36.2%)	0.882
Family history of AS	36 (25.3%)	10 (17.2%)	0.216
Pure axial involvement	29 (20.4%)	13 (22.4%)	0.754
BMI (kg/m²)	24.81 ± 3.72	25.80 ± 3.87	**0.02**
BASMI	4.63 ± 2.22	3.43 ± 2.27	**0.001**
mSASSS	22.54 ± 23.34	10.64 ± 17.2	**<0.0001**
ASDAS-Sed. rate	2.11 ± 1.05	2.42 ± 1.04	**0.036**
HAQ-S	1.03 ± 0.61	1.11 ± 2.33	**0.03**
Sed. rate (mm)	19.3 ± 19.6	28.35 ± 21.31	**0.001**
Disease duration (years)	17.5 ± 9.43	14.43 ± 8.5	**0.02**
Radiographic sacroilitis			
II	11 (7.7%)	4 (7.0%)	**0.044**
III	66 (46.5%)	38 (65.5%)	
IV	65 (44.0%)	16 (27.6%)	

AS, ankylosing spondylitis; BMI, body mass index; BASMI, Bath ankylosing spondylitis metrology index; ASDAS, Ankylosing spondylitis disease activity score; HAQ-S, Health assessment questionnaire-Spondylitis; mSASSS, modified Stokes ankylosing spondylitis spine score; Sed. rate, blood sedimentation rate; chi-square and Mann–Whitney tests.
The *p* value in bold means statistically significant.

analysis ($p = 0.004$). The most frequent degree of radiographic sacroiliitis was Grade III ($n = 103$; 51.8%), followed by Grades IV (40.5%) and II (7.5%).

Among the patients on TNFα inhibitors, 20 (24.7%) needed to change agents: five (6.2%) due to primary failure (6.2%), six (7.4%) due to secondary failure and 13 (16%) due to adverse events, especially infusion reactions and infection. Moreover, four (2%) of these patients changed TNFα inhibitors for more than two agents.

Anterior uveitis was associated with longer disease duration ($p = 0.002$), but lost its statistical significance in the final model. Hip involvement was associated with chronicity and lower disease activity scores (data not shown). In the final logistic regression model, longer disease duration ($p = 0.039$), a higher BASFI score ($p = 0.027$) and a lower BASDAI score ($p = 0.024$) remained statistically significant.

Patients with longer disease duration had higher mSASSS and BASMI scores as well as a lower ASDAS-Sed. rate score. In the final model, only ASDAS-ESH ($p = 0.015$) remained significant. Longer symptom duration was correlated with higher BASMI, BASFI, HAQ-S and mSSASS scores as well as a lower BASDAI score. In the final model, BASMI ($p < 0.0001$) and BASDAI ($p = 0.016$) remained significant after the multiple adjustments.

When the patients were classified by remission (<1.3), moderate (≥1.3 and <2.1), high (≥2.1 and <3.5) or very high (≥3.5)

Table 4 – Characteristics of patients with ankylosing spondylitis according to positivity for HLA-B27.

Variables	HLA-B27 positive (n = 130)	HLA-B27 negative (n = 67)	p
Ethnicity			
Caucasian	69 (53.1%)	26 (39%)	**0.058**
Non-Caucasian	61 (46.9%)	41 (61.2%)	
Onset			
Adult	103 (82.4%)	55 (87.3%)	0.386
Juvenile	22 (17.6%)	8 (12.7%)	
Arthritis	58 (44.6%)	40 (59.7%)	**0.045**
Enthesite	85 (65.4%)	43 (64.2%)	0.867
Uveitis	56 (43.1%)	17 (25.4%)	**0.015**
Family history of AS	31 (23.8%)	14 (20.9%)	0.640
Radiographic sacroiliitis			
II	9 (6.9%)	6 (8.9%)	
III	59 (45.4%)	44 (65.7%)	
IV	62 (47.7%)	17 (25.4%)	**0.01**
Pure axial involvement	28 (21.5%)	12 (17.9%)	0.549
Disease duration (years)	8.28 ± 6.57	6.39 ± 5.07	**0.039**
TNFα inhibitors	59 (45.4%)	16 (23.9%)	**0.003**

Chi-square and Mann–Whitney tests.
AS, ankylosing spondylitis.
The *p* value in bold means statistically significant.

Table 5 – Characteristics of patients with ankylosing spondylitis according to ethnicity.

Variables	Caucasian (n = 96)	Non-Caucasian (n = 104)	p
Onset			
Adult	78 (81.3%)	90 (86.5%)	
Juvenile	18 (18.7%)	14 (13.5%)	0.308
Arthritis	47 (49%)	52 (0.5%)	0.883
Enthesitis	61 (63.5%)	68 (65.4%)	0.786
Uveitis	34 (35.4%)	40 (38.5%)	0.656
Family history of AS	23 (24%)	23 (22.1%)	0.757
Radiographic sacroilitis			
II	6 (6.2%)	9 (8.6%)	
III	59 (61.4%)	45 (43.3%)	
IV	31 (32.3%)	50 (48.1%)	**0.037**
Pure axial involvement	21 (21.8%)	21 (20.2%)	0.770
BMI (kg/m²)	24.81 ± 3.72	25.8 ± 3.87	**0.048**
BASMI	3.89 ± 2.18	4.65 ± 2.36	**0.023**

AS, ankylosing spondylitis; BMI, body mass index; BASMI, Bath ankylosing spondylitis metrology index.
Chi-square and Mann–Whitney tests.
The *p* value in bold means statistically significant.

Table 6 – Characteristics of patients with ankylosing spondylitis according to hip involvement.

Variables	Hip involvement		*p*
	Yes (*n* = 26)	No (*n* = 174)	
Age (years)	47.7 ± 11.3	42.4 ± 12.7	**0.035**
Time since diagnosis (years)	10.3 ± 7.9	7.2 ± 5.9	**0.04**
Disease duration (years)	23.4 ± 9.6	15.6 ± 8.8	**<0.0001**
BASDAI	1.20 ± 1.43	2.42 ± 2.06	**0.001**
BASMI	5.55 ± 1.52	4.10 ± 2.33	**0.002**
BASFI	5.03 ± 2.52	3.61 ± 2.48	**0.011**
mSASSS	27.33 ± 24.46	17.77 ± 21.80	**0.035**
ASDAS-CRP	1.55 ± 0.88	2.14 ± 1.09	**0.009**
HAQ-S	1.86 ± 3.72	0.93 ± 0.61	**0.017**

BASDAI, Bath ankylosing spondylitis disease activity index; BASMI, Bath ankylosing spondylitis metrology index; BASFI, Bath ankylosing spondylitis function index; ASDAS, Ankylosing spondylitis disease activity score; mSASSS, modified Stokes ankylosing spondylitis spine score.
Chi-square and Mann–Whitney tests.
The *p* value in bold means statistically significant.

disease activity based on the ASDAS score, the following frequencies were found: 14% (*n* = 28), 31.5% (*n* = 63), 37% (*n* = 74) and 17.5% (*n* = 35), respectively.

The BASDAI score was positively correlated with BASFI, ASDAS-Sed. rate, ASDAS-CRP, Sed. rate, CRP and HAQ-S and negatively correlated with mSSASS. Following the linear multivariate regression, BASDAI remained significantly correlated with BASFI ($p = 0.001$), HAQ-S ($p = 0.047$), ASDAS-Sed. rate, ASDAS-CRP and Sed. rate ($p < 0.0001$), but not CRP ($p = 0.247$).

Discussion

TLR4 polymorphisms 399 and 299 were not more frequent in Brazilian patients with AS in comparison to the health controls because there were no statistical differences between patient and control groups. The influence of TLR4 polymorphisms in the etiopathogenesis of infections, especially by Gram-negative bacteria, is well known,[19] as such polymorphisms result in a phenotype that is little responsive to endotoxins stemming from the infectious process that cause aberrant transduction of the signal in the presence of microorganisms. However, a number of authors have recently questioned the influence of polymorphisms of this receptor on the progression, severity and outcome of infections, suggesting that factors related to the host are more important than polymorphisms *per se*.[20,21]

Bacterial or intracellular components can initiate the inflammatory process and trigger sensitization to an endogenous antigen through molecular mimicry, persistently activating adaptive innate immunity and perpetuating the inflammatory process. The trigger may not necessarily be pathogenic, but rather makes of part of the normal resident microbiota and can culminate in the development of diseases, such as AS, in genetically susceptible individuals.[2]

There is some evidence of an association between TLR polymorphisms (299 and 399) and AS,[6,22,23] but this association has not been confirmed in other studies.[24–26] The same is true for Asp896Gly.[27] The S180L polymorphism of an adaptor protein of TLR2 and 4 (TIRAP), which has demonstrated to play a protective role against the occurrence of systemic lupus erythematosus, has also demonstrated no association with AS.[28]

Kyo et al. found that mutant mice (C3H/Hej) for TLR4 did not develop arthritis after the intra-joint injection of LPS from *E. coli*, unlike the wild group.[29] This has raised the hypothesis that the mutation in TLR4 may diminish the intensity of the innate immune response, playing a protective role rather than promoting autoimmunity.

The present data show that the 299 and 399 polymorphisms of TLR4 are not genetic factors of greater susceptibility to AS, which is in agreement with a recent meta-analysis involving data compiled from nine studies on this topic.[30] Moreover, it is important to point out that one of the largest genetic studies (The Australo-Anglo-American Spondyloarthritis Consortium), involving more than two thousand patients of European descent with AS, also found no association between TLRs and susceptibility to the disease.[9] According to the authors cited, the main associations occurred with two desert genes (2p15 and 21q22) as well as with IL-23R, IL-1R2, ANTXR2 and ERAP-1.

The expression of TLR4 is related to the interface between the immune system and the environment as well as acute and chronic inflammatory responses in patients with AS,[31–33] but polymorphisms doesn't seem to be associated with a greater risk of developing the disease.[30] Population-based studies involving healthy individuals indicate heterogeneity in the geographic distribution of these polymorphisms. The frequency ranges from 4 to 10% in Caucasians[34] in the present cohort, while a frequency of 16% is reported among Africans.[35] There are no reports of this polymorphism in Asians.[25]

Considering that Brazilian population is highly mixed with multiple ethnic groups, we cannot assure similar results if patients from other parts of the country, particularly no European background, had been included.

One cannot discard the possibility of a weak association that may be representative in a larger sample size than that employed in the present study. However, a study carried out in the United Kingdom involving more than 500 patients with AS also found no association.[6] The same has been true for other ethnicities, such as Hungarian, Finnish, Korean, Canadian and Dutch populations. Another point to consider is the low prevalence of homozygous genotypes for the TLR4 polymorphisms in the present cohort, which is similar to findings described in other populations.

It has been demonstrated that transgenic rats for HLA-B27 do not develop inflammation of the entheses or intestines, although these animals develop genital and skin lesions under sterile conditions, demonstrating the role of infectious agents as an inflammatory trigger.[36] Moreover, the participation of the environment is a determinant in the emergence of the disease, as only two to five percent of the population positive for HLA-B27 develops AS. Based on this fact, the interaction between HLA-B27 and TLR4 polymorphisms was investigated

in the present sample, but was not demonstrated, as no significant differences in the polymorphisms were found between the patients and healthy controls. Moreover, the number of patients with polymorphisms was relatively small.

Regarding clinical aspects, males exhibited more severe axial radiographic damage and longer disease duration than females, which is similar to findings reported in another Brazilian cohort[37] as well as in other populations, although with no significant gender difference regarding peripheral involvement.[38] However, these findings should be interpreted with caution, since the only significant differences after controlling for confounding variables, especially disease duration and age, were BASMI score, which was higher among males, and BMI, which was higher among females.

Miscegenation likely contributed to the two peculiarities found in the present sample. The first was the lesser frequency of positivity for HLA-B27 (66%) in comparison to another Brazilian cohort (78.2%)[37] and studies involving Caucasian populations (more than 90%).[39] Fifty-two percent of the present cohort was non-Caucasian, which better reflects the Brazilian population in comparison to previous studies (75.5% Caucasian patients).[37] The second peculiarity was the greater peripheral or mixed involvement, which has also been reported in other mixed-race populations.[39,40] Among only the Caucasian patients in the present study, the frequency of positivity for HLA-B27 was 75%, which is very close to the frequency reported in a previous Brazilian cohort[39] as well as that reported in a recent French study.[41]

The patients with high chronicity index scores generally had lower disease activity index scores, such as the association between hip involvement and both a worse BASFI and lower BASDAI score. Likewise, disease duration was correlated with a lower BASDAI and higher BASMI score. Interestingly, the BASDAI score was correlated with the other activity markers (ASDAS-Sed. rate and CRP) as well as worse functionality, but the correlation with CRP, which is one of the most standardized parameters for studying inflammatory activity in patients with AS,[42] was non-significant.

As expected, the presence of HLA-B27 was significantly associated with extra-articular manifestations (uveitis)[43] and lesser peripheral involvement, but no associations were found with the radiographic score or any specific AS assessment tool, including activity, function and mobility. This finding reflects the more recent knowledge that this genetic aspect is not an associated factor of a poorer prognosis regarding the formation of new bone.[44]

The prevalence of the juvenile form (16%) was similar to that found in Caucasians (8.6–21%) Turks (13.4%) and other Brazilian cohort (16%)[45] as well as lower than the rates reported for Mexicans (28–54%) and Koreans (41.3%).[46] The inclusion of these patients did not exert an important impact on the clinical, laboratory and imaging outcomes analyzed, which is in agreement with the notion that this subgroup is part of the same spectrum of the disease. In contrast, some authors report lesser axial involvement and a greater proportion of peripheral involvement, especially the knees, in comparison to onset of the disease after the age of 16 years.[37,45,46]

The present study has limitations that should be addressed, such as the sample size for studies on gene polymorphisms and the higher proportion of Caucasian individuals in this cohort, what may not reflect Brazilian AS population. Our data suggest that these TLR4 variations are unlikely to play a role in the etiopathogenesis of AS. This finding, however, does not exclude the possibility that functional abnormalities of the TLRs or other molecules closely associated with the TLRs signaling are important in the pathogenesis of spondylarthropathies.

Funding

Supported by a research grant from the Brazilian fostering agency Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2011/05517-6).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

We thank Rheumatology and Nephrology Division, Federal University of São Paulo (UNIFESP/EPM).

REFERENCES

1. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394–7.
2. Pollanen R, Sillat T, Pajarinen J, Levon J, Kaivosoja E, Konttinen YT. Microbial antigens mediate HLA-B27 diseases via TLRs. J Autoimmun. 2009;32(3-4):172–7.
3. Pacheco-Tena C, Zhang X, Stone M, Burgos-Vargas R, Inman RD. Innate immunity in host–microbial interactions: beyond B27 in the spondyloarthropathies. Curr Opin Rheumatol. 2002;14(4):373–82.
4. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335–76.
5. Leaver SK, Finney SJ, Burke-Gaffney A, Evans TW. Sepsis since the discovery of Toll-like receptors: disease concepts and therapeutic opportunities. Crit Care Med. 2007;35(5):1404–10.
6. Lamarque D, Nhieu JT, Breban M, Bernardeau C, Martin-Garcia N, Szepes Z, et al. Lymphocytic infiltration and expression of inducible nitric oxide synthase in human duodenal and colonic mucosa is a characteristic feature of ankylosing spondylitis. J Rheumatol. 2003;30(11):2428–36.
7. De Rycke L, Kruithof E, Vandooren B, Tak PP, Baeten D. Pathogenesis of spondyloarthritis: insights from synovial membrane studies. Curr Rheumatol Rep. 2006;8(4):275–82.
8. Sparks JA, Costenbader KH. Genetics environment, and gene–environment interactions in the development of systemic rheumatic diseases. Rheum Dis Clin North Am. 2014;40(4):637–57.
9. Reveille JD, Sims AM, Danoy P, Evans DM, Leo P, Pointon JJ, et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. Nat Genet. 2010;42(2):123–7.
10. 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.

11. Rudwaleit M, van der Heijde D, Landewé R, Listing J, Akkoc N, Brandt J, et al. The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part II): validation and final selection. Ann Rheum Dis. 2009;68(6):777–83.
12. 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.
13. Machado P, Landewe R, Lie E, Kvien TK, Braun J, Baker D, et al. Ankylosing Spondylitis Disease Activity Score (ASDAS): defining cut-off values for disease activity states and improvement scores. Ann Rheum Dis. 2011;70(1):47–53.
14. 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.
15. van der Heijde D, Landewe R, Feldtkeller E. Proposal of a linear definition of the Bath Ankylosing Spondylitis Metrology Index (BASMI) and comparison with the 2-step and 10-step definitions. Ann Rheum Dis. 2008;67(4):489–93.
16. Creemers MC, Franssen MJ, van't Hof MA, Gribnau FW, van de Putte LB, van Riel PL. Assessment of outcome in ankylosing spondylitis: an extended radiographic scoring system. Ann Rheum Dis. 2005;64(1):127–9.
17. Jeffreys AJ, Wilson V, Thein SL. Individual-specific 'fingerprints' of human DNA. Nature. 1985;316(6023):76–9.
18. Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques. 2001;31(1):22–4.
19. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1(2):135–45.
20. Draisma A, Dorresteijn M, Pickkers P, van der Hoeven H. The effect of systemic iNOS inhibition during human endotoxemia on the development of tolerance to different TLR-stimuli. Innate Immun. 2008;14(3):153–9.
21. Jessen KM, Lindboe SB, Petersen AL, Eugen-Olsen J, Benfield T. Common T.N.F-alpha, IL-1 beta, PAI-1, uPA, CD14 and TLR4 polymorphisms are not associated with disease severity or outcome from Gram negative sepsis. BMC Infect Dis. 2007;7:108.
22. McCormack WJ, Parker AE, O'Neill LA. Toll-like receptors and NOD-like receptors in rheumatic diseases. Arthritis Res Ther. 2009;11(5):243.
23. Snelgrove T, Lim S, Greenwood C, Peddle L, Hamilton S, Inman R, et al. Association of toll-like receptor 4 variants and ankylosing spondylitis: a case-control study. J Rheumatol. 2007;34(2):368–70.
24. Gergely P Jr, Blazsek A, Weiszhar Z, Pazar B, Poor G. Lack of genetic association of the Toll-like receptor 4 (TLR4) Asp299Gly and Thr399Ile polymorphisms with spondylarthropathies in a Hungarian population. Rheumatology (Oxford). 2006;45(10):1194–6.
25. Na KS, Kim TH, Rahman P, Peddle L, Choi CB, Inman RD. Analysis of single nucleotide polymorphisms in Toll-like receptor 4 shows no association with ankylosing spondylitis in a Korean population. Rheumatol Int. 2008;28(7):627–30.
26. Adam R, Sturrock RD, Gracie JA. TLR4 mutations (Asp299Gly and Thr399Ile) are not associated with ankylosing spondylitis. Ann Rheum Dis. 2006;65(8):1099–101.
27. van der Paardt M, Crusius JB, de Koning MH, Morre SA, van de Stadt RJ, Dijkmans BA, et al. No evidence for involvement of the Toll-like receptor 4 (TLR4) A896G and CD14-C260 T polymorphisms in susceptibility to ankylosing spondylitis. Ann Rheum Dis. 2005;64(2):235–8.
28. Cantaert T, Stone MA, ter Borg M, Mogg R, De Vries N, Wilson AG, et al. A functional polymorphism of TIR-domain-containing adaptor protein is not associated with axial spondyloarthritis. Ann Rheum Dis. 2008;67(5):720–2.
29. Kyo F, Futani H, Matsui K, Terada M, Adachi K, Nagata K, et al. Endogenous interleukin-6, but not tumor necrosis factor alpha, contributes to the development of toll-like receptor 4/myeloid differentiation factor 88-mediated acute arthritis in mice. Arthritis Rheum. 2005;52(8):2530–40.
30. Xu WD, Liu SS, Pan HF, Ye DQ. Lack of association of TLR4 polymorphisms with susceptibility to rheumatoid arthritis and ankylosing spondylitis: a meta-analysis. Joint Bone Spine. 2012;79(6):566–9.
31. De Rycke L, Vandooren B, Kruithof E, De Keyser F, Veys EM, Baeten D. Tumor necrosis factor alpha blockade treatment down-modulates the increased systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondylarthropathy. Arthritis Rheum. 2005;52(7):2146–58.
32. Assassi S, Reveille JD, Arnett FC, Weisman MH, Ward MM, Agarwal SK, et al. Whole-blood gene expression profiling in ankylosing spondylitis shows upregulation of toll-like receptor 4 and 5. J Rheumatol. 2011;38(1):87–98.
33. Yang ZX, Liang Y, Zhu Y, Li C, Zhang LZ, Zeng XM, et al. Increased expression of Toll-like receptor 4 in peripheral blood leucocytes and serum levels of some cytokines in patients with ankylosing spondylitis. Clin Exp Immunol. 2007;149(1):48–55.
34. Carvalho A, Marques A, Maciel P, Rodrigues F. Study of disease-relevant polymorphisms in the TLR4 and TLR9 genes: a novel method applied to the analysis of the Portuguese population. Mol Cell Probe. 2007;21(4):316–20.
35. Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, et al. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci USA. 2006;103(1):177–82.
36. Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernandez-Sueiro JL, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med. 1994;180(6):2359–64.
37. Sampaio-Barros PD, Bertolo MB, Kraemer MH, Neto JF, Samara AM. Primary ankylosing spondylitis: patterns of disease in a Brazilian population of 147 patients. J Rheumatol. 2001;28(3):560–5.
38. Lee W, Reveille JD, Davis JC Jr, Learch TJ, Ward MM, Weisman MH. Are there gender differences in severity of ankylosing spondylitis? Results from the PSOAS cohort. Ann Rheum Dis. 2007;66(5):633–8.
39. Gallinaro AL, Ventura C, Sampaio Barros PD, Goncalves CR. Spondyloarthritis: analysis of a Brazilian series compared with a large Ibero-American registry (RESPONDIA group). Rev Bras Reumatol. 2010;50(5):581–9.
40. Kohem CL, Bortoluzzo AB, Gonçalves CR, Braga da Silva JA, Ximenes AC, Bértolo MB, et al. Profile of the use of disease modifying drugs in the Brazilian Registry of Spondyloarthritides. Rev Bras Reumatol. 2015;55(1):48–54.
41. Costantino F, Talpin A, Said-Nahal R, Goldberg M, Henny J, Chiocchia G, et al. Prevalence of spondyloarthritis in reference to HLA-B27 in the French population: results of the GAZEL cohort. Ann Rheum Dis. 2015;74:689–93.
42. Machado P, Landewe R. Spondyloarthritis. Is it time to replace Basdai with Asdas? Nat Rev Rheumatol. 2013;9(7):388–90.
43. Khan MA. Update: the twenty subtypes of HLA-B27. Curr Opin Rheumatol. 2000;12(4):235–8.
44. Poddubnyy D, Haibel H, Listing J, Marker-Hermann E, Zeidler H, Braun J, et al. Baseline radiographic damage, elevated acute-phase reactant levels, and cigarette smoking status predict spinal radiographic progression in early axial spondylarthritis. Arthritis Rheum. 2012;64(5):1388–98.

45. Duarte AP, Marques CDL, Bortoluzzo AB, Gonçalves CR, da Silva JA, Ximenes AC, et al. Epidemiologic profile of juvenile-onset compared to adult onset spondyloarthritis in a large Brazilian cohort. Rev Bras Reumatol. 2014;54(6):424–30.

46. Baek HJ, Shin KC, Lee YJ, Kang SW, Lee EB, Yoo CD, et al. Juvenile onset ankylosing spondylitis (JAS) has less severe spinal disease course than adult onset ankylosing spondylitis (AAS): clinical comparison between JAS and AAS in Korea. J Rheumatol. 2002;29(8):1780–5.