Study of the frequency of HLA-DRB1 alleles in Brazilian patients with rheumatoid arthritis

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ABSTRACT

The HLA-DRB1 alleles encoding an amino acid sequence (QKRAA/QRRAA/RRRAA) at position 70 74 of the third hypervariable region of the β1 chain of the HLA-DRB1 gene, called shared epitope (SE), are associated with increased susceptibility to and severity of rheumatoid arthritis (RA) in different populations. **Objective:** To determine the frequency of HLA-DRB1 alleles in Brazilian patients with RA and their association with rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA). **Methods:** Four hundred and twelve patients with RA (ACR 1987) and 215 controls were included. HLA-DRB1 typing was performed by use of polymerase chain reaction (PCR) with specific primers and hybridization with sequence-specific oligonucleotide probe (SSOP). ACPA was measured by use of the ELISA technique and RF by nephelometry. The statistical analysis comprised the chi-square and Student t tests and logistic regression. **Results:** HLA-DRB1*04:01, *04:04, *04:05 alleles were associated with RA ($P < 0.05$); despite the wide confidence interval, it is worth noting the association between the DRB1*09:01 allele and RA ($P < 0.05$). HLA-DRB1 SE+ alleles were observed in 62.8% of the patients and in 31.1% of controls (OR 3.62; $P < 0.001$) and were associated with ACPA (OR 2.03; $P < 0.001$). DRB1-DERAA alleles showed a protective effect against RA (OR 0.42; $P < 0.001$). **Conclusion:** In a sample of Brazilian patients with RA, most of whom of mixed heritage, HLA-DRB1 SE+ alleles were associated with susceptibility to disease and presence of ACPA.

Keywords: HLA-DRB1, shared epitope, rheumatoid arthritis, genetic polymorphism, immunogenetics.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by the inflammatory involvement of the synovial membrane of joints, leading to bone and cartilage destruction. Its prevalence in the adult population worldwide ranges from 0.5% to 1%, being 0.46% in Brazil.[1] Its peak incidence is between the fourth and sixth decades, and RA is three times more frequent in women than in men.[2]

Although the etiology of RA remains unknown,[3] several studies have suggested that a combination of genetic and environmental factors is involved. The genetic factor accounts for approximately 60% of the susceptibility to RA.[4] Although the role played by heredity has not been completely

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understood, the major risk factor, responsible for 30% to 50% of the genetic component, seems to be related to human leukocyte antigens (HLA). The HLA is located in the major histocompatibility complex (MHC) present in the short arm of chromosome 6 (6p21.3).[5] A group of alleles in the HLA-DRB1 locus (DRB1*01:01, DRB1*01:02, DRB1*04:01, DRB1*04:04, DRB1*04:05, DRB1*04:08, DRB1*04:10, DRB1*10:01, DRB1*14:02) encodes a shared amino acid sequence (QKRAA/QRRAA/RRRAA, where Q = glutamine; K = lysine; R = arginine; and A = alanine), located in the peptide binding site, at position 70-74 of the third hypervariable region of the HLA-DR molecule, a sequence called shared epitope (SE).[6]

The SE is believed to be involved in the pathogenesis of RA, because it serves as a binding site in the process of arthritogenic peptide presentation to $CD4^+$ T cells involved in the immune inflammatory response of this disease. Moreover, the SE can be involved in the process of inducing some B cells to differentiate into plasma cells, leading to the formation of anti-citrullinated peptide antibodies (ACPA).[7] In addition to its role in susceptibility to RA, alleles containing the SE sequences (mainly homozygous) are associated with more severe forms of disease and extra-articular manifestations,[8] and also with the presence of erosive disease.[9,10]

Studies conducted in several ethnic groups have shown the existence of considerable variations regarding the association of HLA-DRB1 alleles with susceptibility to RA.[11] The following associations of the HLA-DRB1 alleles, susceptibility to RA and ethnic groups have been reported: HLA-DRB1*04:01 and *04:04 alleles in Caucasians of Northern Europe and the United States;[12] DRB1*04:05 alleles in Koreans, Japanese, and Chinese;[13,14] DRB1*01:01 and *10:01 alleles in Greeks, Spaniards, and Israeli Jews;[15,16] DRB1*14:02 alleles in Native American, Peruvian[17,18] and Ecuadorean[19] Indians; and DRB1*04:04 alleles in Colombians and Argentians.[20,21] In Brazil, Bertolo et al.[22] have reported an association of RA with DRB1*01:01 and *01:02 in 65 Caucasians patients. More recently, Louzada-Junior et al.[23] have reported an association of the disease with the DRB1*04:01, *04:04, *04:05, *01:01, and *10:01 alleles in 140 patients, most of whom of Caucasian ethnicity.

However, the HLA-DRB1 alleles, with a common amino acid sequence, DERAA (D = aspartic acid, E = glutamic acid, R = arginine, A = alanine), expressed in the DRB1*01:03, *04:02, *11:02, *11:03, *13:01, 13:02 and *13:04 alleles, seem to be associated with a lower risk for developing RA, despite the presence of SE. The presence of these alleles also seems to protect against severe erosive disease, even in patients with ACPA.[24,25]

The main immunological markers of RA, rheumatoid factor (RF) and ACPA, also seem to be involved in the pathogenesis of rheumatoid synovitis. High titers of both markers have been associated with more aggressive and erosive disease.[26,27]

Some studies in patients with RA have shown controversial results regarding the association with SE and positivity for RF.[28,29] At the same time, there seems to be an association of SE+ alleles only in patients with RA who are positive for ACPA,[30] and this association would be more intense with ACPA than with RA itself.[31] This association suggests that the SE alleles could influence the antigen presentation, leading to the production of ACPA. More recently, the risk carried by smoking, the main environmental factor, was observed to be particularly high in individuals with HLA-DR SE+ alleles and ACPA.[32]

Considering the diversity of the results in literature regarding the association of HLA-DRB1 alleles with RA in different ethnicities, this study aimed at determining the association between these alleles and RA, including the presence of RF and/or ACPA, in Brazilian patients of highly mixed heritage.

MATERIAL AND METHODS

Patients and control

This was a case-control study with RA patients, all of whom met at least four of the seven criteria for the diagnostic classification of RA established by the American College of Rheumatology (ACR).[33] The patients selected had no other autoimmune diseases and were regularly followed-up at the outpatient clinics of RA of the discipline of Rheumatology of the Hospital Universitário Pedro Ernesto of the Universidade Estadual do Rio de Janeiro (UERJ) and the Hospital São Paulo of the Universidade Federal de São Paulo (UNIFESP), from October 2007 to August 2009. The control group comprised volunteer bone marrow donors from donation campaigns carried out in different neighborhoods of the city of Rio de Janeiro from May 2008 to November 2009. The donors were of both genders, had neither current nor past complaint of arthritis, and their ages ranged from 18 to 55 years. Individuals with a family history of RA or other autoimmune diseases in first-degree relatives were excluded from the study. Individuals older than 30 years were selected to minimize the analysis bias regarding a possible future diagnosis of this disease in the group.

After being informed about the nature of the study and having provided written informed consent, the participants underwent a clinical interview with a standardized form for both groups. Demographic data were collected for both groups, and clinical characteristics for the group of patients. Both groups underwent blood collection for HLA typing. The

group of patients also underwent autoantibody detection (RF and ACPA).

The groups were paired for sex and ethnicity. The ethnic origin was attributed by the same investigator, after asking the patients and volunteer donors about their ancestors. Black or Caucasian ethnicity was attributed to an individual whose all four grandparents were of Black or Caucasian ethnicity, respectively; the mixed ethnicity was attributed to an individual when at least one of his/her grandparents had a different ethnicity. No Asian descendant was included in the study to avoid a selection bias.

Individuals who did not have sufficient DNA or serum amount for an adequate analysis of HLA typing or autoantibody measurement in the two groups were excluded. Individuals whose HLA typing was ambiguous were not included.

HLA-DRB1 typing

The HLA-DRB1 alleles were determined for all RA patients and controls. The HLA typing was performed with the polymerase chain reaction (PCR) using specific primers and hybridization with the specific sequence oligonucleotide probe (SSOP) (One Lambda Inc., Canoga Park, CA, USA).

Definition of HLA-DRB1 alleles posing risk for RA

All individuals typed for the HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *10:01 and *14:02 alleles were considered as having the SE (SE+), which could be present in a single (SE+/-) or double dose (SE+/+). Risk associations for RA between HLA-DRB1 alleles were performed based on genotypic combinations established in the absence of the SE (-/-), and in the presence of a single (SE+/-) or double dose of the SE (SE+/+). The HLA-DRB1 alleles were also associated with the serological characteristics of RA patients.

Definition of the protective HLA-DRB1 alleles against RA

All individuals typed for the HLA-DRB1*01:03, *04:02, *11:02, *11:03, *13:01, *13:02 and *13:04 alleles were considered as having the DERAA sequence (protective), which could be present in a single or double dose.

Individuals with typing comprising alleles that did no belong to the sequence of SE or DERAA were defined as X, which could be present in a single or double dose.

Thus, the following six groups of individuals, according to the presence of DRB1 alleles, were analyzed:
Group A: double dose (homozygous) of SE allele (SE/SE);
Group B: single dose (heterozygous) of SE allele (SE/X);
Group C: single dose of SE and DERAA alleles (SE/DERAA);
Group D: lack of SE and DERAA alleles (X/X);
Group E: single dose of DERAA allele (DERAA/X);
Group F: double dose of DERAA allele (DERAA/DERAA).

Measuring RF and ACPA

The ACPA was measured using the second generation QUANTA Lite™ CCP IgG ELISA (INOVA Diagnostics, Inc., San Diego, USA) according to the instructions of the manufacturer. The IgM RF was quantified using the nephelometry technique (Dade Behring Marburg GmbH – Germany). Regarding IgM RF, values > 20 UI/mL were considered positive, while values > 100 UI/mL were considered high titers. Regarding ACPA, titers > 20 U/mL were considered positive, while those > 60 U/mL were considered high.

Statistical analysis

Data were assessed using the Epi Info 6 program. The association analysis between the categorical variables was performed by chi-square test (Yates correction) or Fisher exact test. Quantitative variables with normal distribution was assessed by Student *t* test for independent samples or Mann-Whitney test, and of analysis of variance. $P < 0.05$ was considered statistically significant. Logistic regression was used to categorize the risk of the association with HLA-DRB1 alleles.

RESULTS

This study included 412 (95%) of 430 patients with RA and 215 (74%) controls of 290 volunteer bone marrow donors interviewed. Gender and ethnicity were similar in both groups ($P = 0.722$ and $P = 0.552$). The other demographic, clinical, and laboratory data of RA patients and controls are shown in Table 1.

Association of HLA-DRB1 allele groups with RA

Table 2 shows the distribution of the frequencies of HLA-DRB1 allele groups and their association with susceptibility to and protection against RA in 412 patients with RA and 215 volunteers in the control group. The HLA-DRB1*04 (OR = 2.69), *09 (OR = 5.19) and *14 (OR = 2.26) alleles were associated with susceptibility to RA ($P < 0.05$), while the DRB1*11 (OR = 0.45) and *13 (OR = 0.53) alleles were associated with protection against the development of RA ($P < 0.05$). Similarly, when using high resolution HLA-DRB1 allele typing (Table 3), the analysis of allele frequencies showed that the DRB1*04:01

Table 1
Demographic, clinical, and laboratory characteristics of RA patients and controls

Variable	RA (n = 412)	Controls (n = 215)	P
Age (mean ± SD, years)	51.8 ± 11.5/52.5	42.6 ± 5.4	< 0.001
Female gender (%)	376 (90.8)	198 (92.1)	0.722
Ethnicity			0.552
Mixed (%)	272 (66)	147 (68.4)	
White (%)	111 (27)	58 (27)	
Black (%)	29 (7)	10 (4.7)	
Duration of disease (mean ± SD, years)	9.2 ± 7.5	—	
RF+ (%)	257 (62.5)	—	
RF titers (mean ± SD, UI/mL)	288 ± 668	—	
ACPA+ (%)	294 (71.3)	—	
ACPA titers (mean ± SD, U/mL)	135 ± 51.5	—	

RA: rheumatoid arthritis; ACPA: anti-citrullinated peptide antibodies; RF: rheumatoid factor; SD: standard definition.

Table 2
Frequencies of HLA-DRB1 allele groups (low resolution) in RA patients and controls

Allele groups	RA = 412, n(%)	Controls = 215, n(%)	OR	95% CI	P
*01	113 (14)	45 (10.5)	1.36	0.93-2.00	NS
*03	73 (9)	54 (12.5)	0.68	0.46-1.00	NS
*04	186 (22.5)	42 (10)	2.69	1.86-3.92	0.001
*07	79 (9.5)	52 (12)	0.77	0.52-1.14	NS
*08	38 (5)	20 (5)	0.99	0.55-1.79	NS
*09	29 (3.5)	3 (1)	5.19	1.50-21.49	0.004
*10	27 (3)	14 (3)	1.01	0.50-2.04	NS
*11	53 (6)	57 (13)	0.45	0.30-0.68	0.001
*12	12 (1.5)	9 (2)	0.69	0.27-1.79	NS
*13	85 (10)	77 (18)	0.53	0.37-0.75	0.001
*14	38 (5)	9 (1)	2.26	1.04-5.09	0.038
*15	64 (8)	43 (10)	0.76	0.50-1.16	NS
*16	27 (3)	11 (2.5)	1.29	0.61-2.80	NS
TOTAL	824	430			

RA: rheumatoid arthritis; OR: odds ratio; NS: non-significant.

(OR = 2.76), *04:04 (OR = 3.80), *04:05 (OR = 2.60) and *09:01 (OR = 5.19) alleles were associated with susceptibility to RA (P < 0.05).

Association of the HLA-DRB1 alleles of the SE with susceptibility to RA

Table 4 shows the distribution of RA patients and controls according to the presence of HLA-DRB1 SE+ alleles. A higher frequency of heterozygous (51%) than homozygous (11%) genotypes was observed in the group of patients, being both associated with susceptibility to RA (OR = 2.90, P < 0.001; and OR = 2.27, P < 0.001, respectively). In addition, an increased frequency of SE was observed in patients with RA (62.8%) as compared with controls (31.1%), resulting in OR = 3.59 (P = 0.05).

The logistic regression analysis of data from RA patients and controls with homozygous and heterozygous genotypes HLA-DRB1 SE+ and SE- showed a 3.86-time increased risk

Table 3
Allele frequency of HLA-DRB1 in RA patients and controls

HLA-DRB1 alleles	RA = 824, n(%)	Controls = 430, n(%)	OR	95% CI	P
*01:01	68 (8.3)	26 (6)	1.40	0.86-2.29	NS
*01:02	41 (5)	17 (4)	1.27	0.69-2.36	NS
*01:03	4 (0.5)	2 (0.5)	1.04	0.16-8.22	NS
*03	73 (8.8)	54 (12.5)	0.68	0.46-1.00	NS
*04:01	41 (5)	8 (1.9)	2.76	1.23-6.44	0.010
*04:02	14 (1.7)	5 (1.2)	1.47	0.49-4.70	NS
*04:03	9 (1.1)	5 (1.2)	0.94	0.29-3.23	NS
*04:04	42 (5)	6 (1.4)	3.80	1.53-10.0	0.002
*04:05	48 (5.8)	10 (2.3)	2.60	1.25-5.53	0.007
*04:06	3 (0.4)	1 (0.2)	1.57	0.15-39.2	NS
*04:07	7 (0.8)	3 (0.7)	1.22	0.28-5.97	NS
*04:08	12 (1.5)	0 (0.0)	ND	ND	NS
*04:10	3 (0.4)	0 (0.0)	ND	ND	NS
*04:11	7 (0.8)	4 (0.9)	0.91	0.24-3.72	NS
*07	79 (9.6)	52 (12.1)	0.77	0.52-1.14	NS
*08	38 (4.6)	20 (4.7)	0.99	0.55-1.79	NS
*09:01	29 (3.5)	3 (0.7)	5.19	1.50-21.49	0.004
*10:01	27 (3.3)	8 (1.9)	1.79	0.77-4.31	NS
*11	53 (6.4)	57 (13.2)	0.45	0.30-0.68	0.001
*12	12 (1.4)	9 (2.1)	0.69	0.27-1.79	NS
*13	85 (10.3)	77 (17.9)	0.53	0.37-0.75	0.001
*14:01	20 (2.4)	6 (1.4)	1.76	0.66-4.92	NS
*14:02	18 (2.2)	3 (0.7)	3.18	0.88-13.63	NS
*15	64 (7.8)	43 (10)	0.76	0.50-1.16	NS
*16	27 (3.3)	11 (2.6)	1.29	0.61-2.80	NS

RA: rheumatoid arthritis; OR: odds ratio; NS: non-significant.

for the disease among patients with homozygous genotypes (95% CI 1.84-8.24; P < 0.001), and a 3.54-time greater risk among those with heterozygous genotypes (95% CI 2.40-5.21; P < 0.001).

Association of the HLA-DRB1-DERAA alleles with protection against the development of RA

Assessing the influence of the presence of DERAA alleles, 79 (19%) patients with RA and 78 (36%) controls had HLA-DRB1 alleles encoding DERAA, indicating that their presence protects against RA (OR = 0.42; 95% CI 0.28-0.61; P < 0.001).

The effect of DERAA alleles in the absence of SE alleles was assessed by comparing the D (X/X) group with the E (X/DERAA) and F (DERAA/DERAA) groups. DERAA-positive individuals had a lower risk for RA (OR = 0.56; 95% CI 0.34-0.92; P = 0.021).

The comparison of the B (SE/X) group with the C (SE/DERAA) group revealed that, in the presence of a SE allele, the DERAA allele reduced the susceptibility to RA, although with no statistical significance (OR = 0.60, 95% CI 0.28-1.29; P = 0.216) (Table 5).

Table 4
Individual specificities of HLA-DRB1 associated with RA in Brazilians according to the presence of SE in genotypes

SE and genotype	RA = 412, n(%)	Controls = 215, n(%)	OR	95% IC	P
SE+/SE- alleles	**210 (51)**	**56 (26)**	**2.90**	**1.99-4.23**	**< 0.001**
*01:01	48 (11.6)	18 (8.3)	1.44	0.79-2.65	0.257
*01:02	32 (7.7)	11 (5.1)	1.56	0.74-3.37	0.280
*04:01	23 (5.5)	6 (2.7)	2.06	0.78-5.74	0.167
*04:04	27 (6.5)	4 (1.8)	3.70	1.21-12.54	0.017
*04:05	35 (8.4)	7 (3.2)	2.76	1.15-6.94	0.020
*04:08	8 (1.9)	—	NS	—	0.092
*04:10	3 (0.7)	—	NS	—	0.519
*10:01	21 (5)	7 (3.2)	1.60	0.63-4.20	0.392
*14:02	13 (3.1)	3 (1.3)	2.30	0.61-10.28	0.289
SE+/SE+ alleles	**45 (11)**	**11 (5)**	**2.27**	**1.11-4.77**	**0.023**
*01:01/*01:01	1 (0.2)	1 (0.4)	NS	NS	NS
*01:02/*01:02	1 (0.2)	1 (0.4)	NS	NS	NS
*04:01/*04:01	1 (0.2)	—	NS	NS	NS
*04:04/*04:04	3 (0.7)	—	NS	NS	NS
*04:05/*04:05	1 (0.2)	—	NS	NS	NS
*14:02/*14:02	1 (0.2)	—	NS	NS	NS
*01:01/*04:01	8 (1.9)	2 (0.9)	2.11	0.41-14.50	0.532
*01:01/*04:04	3 (0.7)	—	NS	NS	NS
*01:01/*04:05	3 (0.7)	2 (0.9)	NS	NS	NS
*01:01/*10:01	2 (0.5)	—	NS	NS	NS
*01:02/*04:01	2 (0.5)	—	NS	NS	NS
*01:02/*04:04	3 (0.7)	1 (0.9)	NS	NS	NS
*04:01/*04:05	3 (0.7)	1 (0.9)	NS	NS	NS
*04:01/*10:01	2 (0.5)	—	NS	NS	NS
*04:05/*10:01	2 (0.5)	—	NS	NS	NS
Outros	9 (2.2)	3 (1.3)	1.58	0.39-7.43	0.705
Total EC+	**255 (62.8)**	**67 (31.1)**	**3.59**	**2.49-5.17**	**< 0.001**

RA: rheumatoid arthritis; SE+: positive shared epitope; SE-: negative shared epitope; OR: odds ratio; NS: non-significant.

Association between susceptibility HLA-DRB1 alleles (SE) and the presence of autoantibodies

Regarding the patients with RA, 257 (62.3%) were RF positive, 294 (71.3%) were ACPA positive, of whom 237 (57.5%) were positive for both autoantibodies, and only 57 (13.8%) were positive only for ACPA. A significant association was also observed between RF and ACPA (OR = 20.79; 95% CI 11.49-37.97; P < 0.001).

One hundred and sixty-five (40.04%) patients expressed FR+ and HLA-DRB1 SE+ alleles, and 197 (47.81%) expressed ACPA+ and HLA-DRB1 SE+ alleles. A significant association was observed only between SE alleles and ACPA (Table 6).

The serum levels of RF in patients with SE homozygous genotypes, SE heterozygous genotypes, and genotypes without SE were 177.7 ± 286 IU/mL, 205.3 ± 695 IU/mL, and 157.1 ± 328 IU/mL, respectively. The mean ACPA serum levels, following the same order, were 131.3 ± 66 U/mL, 102.8 ± 71 U/mL, and 83.3 ± 73U/mL, respectively.

Table 5
Frequencies of the susceptibility HLA-DRB1 genotypes for RA (SE) and protection against RA (DERAA) in RA patients and controls

Group	HLA-DRB1 genotype	RA = 412, n(%)	Controls = 215, n(%)
A	**SE/SE**	45 (10.9)	11 (5.1)
B	**SE/X**	175 (43.2)	42 (19.5)
C	**SE/DERAA**	35 (7.7)	14 (6.5)
D	**X/X**	110 (26.6)	84 (39.1)
E	**X/DERAA**	43 (10.4)	57 (26.5)
F	**DERAA/DERAA**	4 (0.9)	7 (3.3)

SE alleles are HLA-DRB1 *01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01, and *14:02. DERAA alleles are HLA-DRB1 *01:03, *04:02, *11:02, *11:03, *13:01, *13:02, and *13:04. X alleles are all other HLA-DRB1 alleles.

OR, 95% CI and P values of the following data: Group B compared with group C: OR 0.60; 95% CI 0.28-1.29; P = 0.216. Group D compared with groups E and F: OR 0.56; 95% CI 0.34-0.92; P = 0.021. Group A plus group B compared with group D: OR 3.17; 95% CI 2.05-4.90; P = 0.00.

Table 6
Frequency of the HLA-DRB1 SE allele and autoantibodies in 412 RA patients

Autoantibodies	SE+, n(%)	SE-, n(%)	OR	95% IC	P
Rheumatoid factor			1.26	0.82-1.94	0.313
Positive	165 (40.04)	92 (22.33)			
Negative	91 (22.08)	64 (15.53)			
ACPA			2.03	1.28-3.31	0.001
Positive	197 (47.81)	97 (23.54)			
Negative	59 (14.32)	59 (14.32)			

SE+: positive shared epitope; SE-: negative shared epitope; ACPA: anti-citrullinated peptide antibodies; OR: odds ratio.

DISCUSSION

Genetic studies initially conducted in twins and later in their relatives have shown a familial predisposition to RA, representing 60% of the total risk for development of disease in the population.[4] Several studies have shown that the most important genetic factor associated with susceptibility to RA is the presence of the HLA-DRB1 allele of MHC, discovered by Stastny et al.[34], and later approached as the SE hypothesis by Gregersen et al.[6]

Modern techniques, such as those used in genome-wide association studies (GWAS) and genetic markers of single nucleotide polymorphism (SNP), have confirmed the significant and preponderant association of these alleles with RA. However, it is worth emphasizing that the association between RA and HLA-DRB1 allele and the SE hypothesis do not explain all the genetic susceptibility resulting from the HLA. Other genes of the HLA, without SE, such as the HLA-DRB1*03:01, DRB1*07:01 and DRB1*09:01 alleles, although less often, have also been associated with higher susceptibility to RA.[35,36]

A second gene associated with the disease is the protein tyrosine phosphatase N22 (PTPN22) gene, present in 8% of RA patients.[32] To a lesser extent, associations with the following alleles have been reported: the signal transducer and activator of transcription 4 (STAT4), cytotoxic T-lymphocyte antigen 4 (CTLA4), macrophage migration inhibitory factor (MIF), and peptidylarginine deiminase type IV (PADI4).[37]

In the present study, 90.8% of the patients were females, similarly to the proportion reported by Bertolo et al.[22] in 2001 (84.6%) and by Louzada-Junior et al.[23] in 2008 (77.8%).

The influence of ethnicity is a fundamental issue when assessing the association of HLA genes with diseases, especially with RA. In addition to being a highly polymorphic system, distinct alleles are associated with RA in different populations. Thus, when studying a population of highly mixed heritage, such as the Brazilian one, the traditional associations of certain ethnicities might not be observed. Aiming at controlling

this variable, every study on the association between HLA and disease requires controls from the same sociogeographic stratus of patients studied to preserve the social and demographic influences in both groups. In addition to assessing a significant sample of RA patients (n = 412), this study also typified healthy individuals (n = 215), whose geographical region and social stratus were equal to those of the patients. Furthermore, individuals of mixed heritage predominated, followed by white and black individuals, resulting in a highly miscegenated sample (mainly descendants from Portuguese, Africans, Indians, Italians, Spaniards, and German), which we believe partially reflects the Brazilian population itself.

In theory, in genetic studies, age would not influence susceptibility. However, because of the presence of HLA-DRB1 SE+ alleles in 12.5% to 35%[23,38] of the healthy population, and the higher incidence of RA between 30 and 55 years,[2] we tried to avoid analysis biases regarding the diagnosis in the control group by including in our control group individuals with more advanced age.

Regarding the HLA-DRB1 and disease association, an increased frequency of the HLA-DRB1*04:01, *04:04 and *04:05 alleles and a positive association with susceptibility to RA have been observed. This result is partially in accordance with those by Louzada-Junior et al.[23] in the predominantly Caucasian Brazilian population, in which, in addition to the association with the previously cited alleles, an association with the HLA-DRB1*01:01 allele has also been reported. However, this differs from the findings of Bertolo et al.,[22] who have reported a significant association only with the HLA-DRB1*01 alleles (OR = 2.8), also assessing Caucasian populations.

Similarly to that observed by Louzada-Junior et al.,[22] the HLA-DRB1*14:02 allele has shown a tendency towards the association with RA, although not significantly (OR = 3.18; 95% CI 0.88-13.63; P = 0.086). Studies with a higher number of individuals might show statistical significance in this association. A study with a mixed Peruvian population (n = 65) has shown that the HLA-DRB1*14:02 allele and RA are associated (OR = 2.74).[18]

The HLA-DRB1*09 alleles, which predominate in black and Indian descendants, present in 6.7% of RA patients and in 1.3% of controls showed a significant association with susceptibility to RA in the heterozygous genotypes of these alleles (OR = 5.19; 95% CI 1.50-21.49; P = 0.004). Despite the wide confidence interval observed, this allele was for the first time associated with susceptibility to RA in the Brazilian population. That same association (HLA-DRB1*09 and RA) was also reported in Chilean patients with RA in 1990.[39] However, a study carried out in Japan with 852 RA patients found only the association of the homozygous HLA-DRB1*09:01 genotype with the disease.[36]

Similarly to the studies by Vignal et al.[35] (OR = 5.04) and Balsa et al.[38] (OR = 1.8) including an ethnically homogeneous population, we also found an association between the set of HLA-DRB1 SE+ alleles and RA (OR = 3.59). This result differs from the one reported by Teller et al.[40] in a study including RA patients and Hispanic-American controls, in which no association between these alleles and the disease was observed, suggesting that the hypothesis of the SE might not be applied in studies with non-mixed populations.

In the present study, an increased frequency of the heterozygous HLA-DRB1 SE genotypes (51%) can be observed when compared with RA patients and controls with homozygous genotypes (11%). In a study by del Rincon et al.,[41] heterozygous SE genotypes were observed in 52% of RA patients, and homozygous SE genotypes in 22%. However, Balsa et al.[38] have reported 29.8% and 14% of heterozygous and homozygous patients, respectively. In this study, after logistic regression analysis of homozygous and heterozygous HLA-DRB1 SE+ genotypes, patients with SE, regardless of being homozygous (OR = 3.86) or heterozygous (OR = 3.54), showed an increased risk but of similar OR for the development of RA. This result is not in accordance with the study by del Rincon et al.,[41] in which the assessment of 141 Mexican patients with RA showed a higher risk for homozygous SE genotypes (OR = 21.53) as compared with heterozygous SE genotypes (OR = 1.84).

Regarding the protective HLA-DRB1 alleles against RA, HLA-DRB1 alleles encoding the DERAA amino acid sequence were observed to associate with a lower risk for developing RA (OR = 0.42), similarly to that reported by Louzada-Junior et al.[23] in 2008 (OR = 0.49). Carrier et al.[25] have assessed patients with recent-onset polyarthritis, and have reported that DERAA alleles were neither associated with the production of autoantibodies, nor showed a protective effect on the development of RA (OR = 0.30). More recently, Balsa et al.[38] have reported that these alleles provide protection against RA only in the presence of circulating ACPA (OR = 0.58). Another study has shown that DERAA alleles, in addition to protecting against RA, are associated with less severe illness.[24]

We observed a higher frequency of RF (62%) and ACPA (71%) positivity as compared with a group of Brazilian patients with initial RA,[42] whose frequencies were approximately 50%. This result can be due to the fact that our group of patients has a longer duration of disease (approximately nine years). The HLA-DRB1 SE alleles were related to the RF and ACPA positivity, but only the association with the presence of ACPA was significant (Table 6). In 2005, Irigoyem et al.[29] reported a strong

association between ACPA and SE alleles, independently of the presence of RF (OR = 5.8; 95% CI 4.1-8.3; P < 0.001; and OR = 3.1; 95% CI 1.8-5.3; P < 0.001). Similarly to that observed by Balsa et al.,[38] this study showed that homozygous HLA-DRB1 SE genotypes had higher serum levels of ACPA as compared with those of the heterozygous genotypes and the patients without SE. In contrast, the serum levels of RF did not differ in patients with and without the SE alleles. We observed that of 27 patients with the HLA-DRB1*09 allele, 88.8% had ACPA and 85% had RF, suggesting that mechanisms other than SE would be implicated in the genetic risk for developing RF and ACPA.

Briefly, this study, with a population sample of predominantly mixed heritage and more representative of the Brazilian population, evidenced that the HLA-DRB1*04:01, *04:04, and *04:05 alleles were associated with an increased susceptibility to RA, and also emphasized the association with the DRB1*09 allele in these patients. Our results support the association between DRB1 SE alleles and susceptibility to RA and ACPA previously reported in studies with genetically homogeneous population samples. In addition, we showed that the presence of the SE allele, either in a single or double dose, acted as an independent risk factor for the disease, and the presence of the DERAA alleles showed a protective effect. Furthermore, the HLA-EC alleles were associated with higher positivity and higher serum levels of ACPA. Although the Brazilian Society of Rheumatology guidelines for the diagnosis of RA established in 2011[43] recommend that the HLA-EC measurement should not be a daily routine test in the management of patients suspected of having RA because of its high cost, our study confirms its importance in establishing the risk for and protective factors against the development of RA.

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