*HLA-B*51* and its main subtypes in Brazilian patients with Behçet's disease

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Received on February 17, 2020; accepted in revised form on June 16, 2020.

Clin Exp Rheumatol 2020; 38 (Suppl. 127): S53-S59.

Key words: Behçet's syndrome, HLA, MHC, immunogenetics

Funding: the authors received grants from Fleury Health and Medicine Laboratories to conduct the study.

Competing interests: J.M.F.M. Belem received a bursary from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The other co-authors have declared no competing interests.

ABSTRACT

Objective. *This study aimed to evaluate the frequency of HLA-B*51 and its subtypes in Brazilian patients with Behçet's disease (BD) and in healthy controls (HC) and to assess possible associations with disease manifestations.*

Methods. *A cross-sectional study with sequential BD patients and HC. HLA-B*51 presence was determined by sequence-specific polymerase chain reaction (SSP-PCR) and HLA-B*51 subtypes by the Sanger sequencing method.*

Results. *Eighty-three BD patients and 258 HC were evaluated. HLA-B*51 was found in 30.1% of DB patients and in 15.5% of HC (p=0.003). The most prevalent subtypes in DB patients were HLA-B*51:01 (60.0%), HLA-B*51:08 (20.0%), HLA-B*51:22 (8.0%), HLA-B*51:29 (8.0%) and HLA-B*51:02 (4.0%), while HLA-B*51:01 (77.5%) and HLA-B*51:55 (7.5%) were the most prevalent in HC. HLA-B*51 was less frequently found in patients with neurologic involvement (8.0% vs. 29.3%; p=0.034) while HLA-B*51:01 was more observed in patients with ocular involvement (93.3% vs. 60.3%; p=0.014). No BD patient with neurologic or vascular involvement presented HLA-B*51:01. HLA-B*51:08 was more frequent in patients with vascular manifestations (60.0% vs. 15.4%; p=0.012). In multivariate analysis, HLA-B*51 was an independent risk factor for BD (OR=2.410; 95%CI: 1.332–4.361; p=0.004) and HLA-B*51:08 had an independent association with vascular manifestations of BD (OR=14.843; 95%CI: 1.550–142.115; p=0.019).*

Conclusion. *The prevalence of HLA-B*51 is higher in Brazilian BD patients compared to HC, and it is a risk factor for BD. The HLA-B*51:08 subtype was independently associated with vascular manifestations of BD.*

Introduction

Behçet's disease (BD) is a systemic inflammatory disorder classified as a variable vessel vasculitis and characterised by recurrent oral and genital ulcers, skin lesions and ocular, articular, intestinal, neurological, and vascular involvement (1, 2). Epidemiologic studies identify that cases of BD cluster along the ancient Silk Road, which extends from China and Japan in the far East to the Mediterranean Sea in the Middle East, where genetic factors such as *HLA-B*51* are spread at an increased frequency (3, 4). The highest prevalence of BD (20-421/100,000) is observed in Turkey, whereas in Asian countries it ranges from 8.6 to 80.0/100,000 (5-13). The prevalence of BD is lower in the United States and Northern Europe (0.3-5.2/100,000 inhabitants), but it rises from the North to the South of the European continent (14-20). To date, no studies have addressed the epidemiology of BD in Brazil.

In addition to the epidemiologic features of BD, the genetic predisposition for BD is also suggested by the higher concordance for BD in monozygotic twins compared to dizygotic twins (21). Indeed, the immunopathogenesis hypothesis for BD speculates that the disease process may be triggered by microbiological factors in genetically susceptible individuals (4, 22). The genetic approach identified associations between distinct genetic markers and BD, and the *HLA-B*51*, a split product of *HLA-B*5,* is the strongest susceptibility factor for BD. This evidence has been supported by genome-wide association studies (GWAS) (23-27). It is estimated that carriers of the *HLA-B*5/B*51* have a pooled odds ratio for the development of BD of 5.78 (95% confidence interval: 5.00-6.67) compared with non-carriers and the population-attributable risk for BD concern-

ing the presence of *HLA-B*5/B*51* within different geographic areas was estimated at 32-52% (28). A meta-analysis found that the *HLA-B*5/B*51* allele is more commonly observed in males with BD and this allele increased the chance of genital ulcers, ocular and cutaneous manifestations in these patients, as well as a decreased frequency of gastrointestinal involvement. Nevertheless, this genetic association had an only modest phenotype-modifying effect on BD (29).

Recently, a hospital-based survey has shown BD as the most prevalent form of vasculitis in Brazil (30), but the prevalence of *HLA-B*51* and its main subtypes are not yet known in Brazilian BD patients. Therefore, this study aims to evaluate the prevalence of *HLA-B*51* and its subtypes in BD patients and healthy controls (HC) from the city of São Paulo. Possible associations between *HLA-B*51* and its subtypes with clinical manifestations of BD were also analysed.

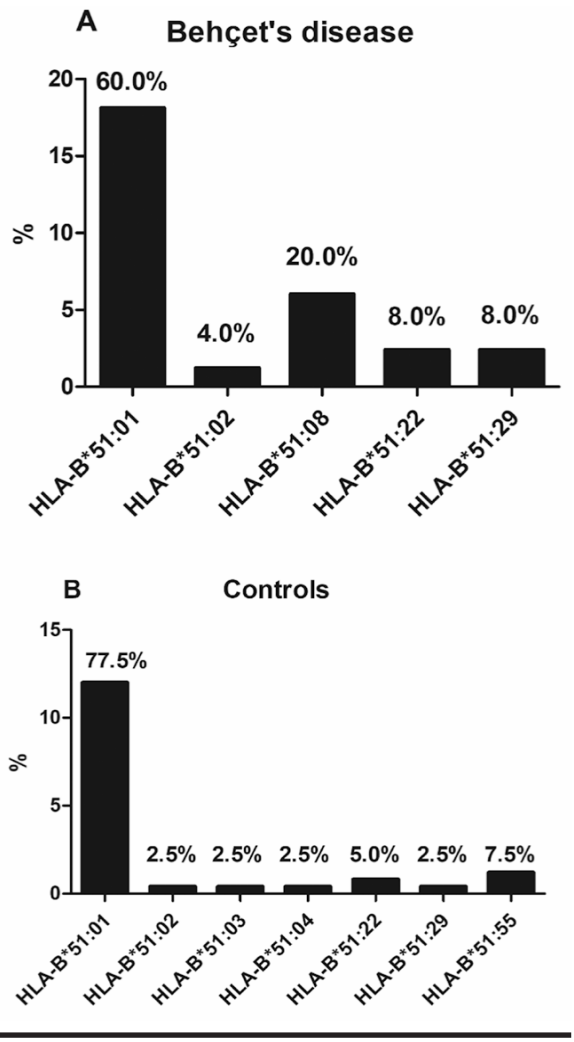

Fig. 1. *HLA-B*51* subtypes in patients with Behçet's disease and heathy controls. The percentage of each subtype is related to the number of *HLA-B*51* positive individuals in Behçet's disease group and control group, respectively.

Methods

Patients and healthy controls

A cross-sectional study was performed, including BD patients and HC. Consecutive BD patients were recruited from the Vasculitis Outpatient Clinic at the Universidade Federal de São Paulo, Escola Paulista de Medicina (UNIFESP-EPM), Brazil using the following inclusion criteria: the fulfillment of the International Study Group for BD (ISG) (31) diagnosis criteria or the International Criteria for BD (ICDB) (32) and written informed consent. Individuals in the HC group were recruited among blood donors and patients who underwent a clinical interview and laboratory tests at the Central Laboratory of the University Hospital. Inclusion criteria in the HC group were the absence of manifestations of BD, no previous history of systemic autoimmune diseases, and written informed consent. HC were preferentially invited to participate in the study if they were older than 45 years (*i.e.* above the mean age at BD presentation in our patients) (33). The study was conducted in accordance with the declaration of Helsinki, and the institutional review board approved the study (protocol no. 719,765-14).

Disease-related variables

Information about previous manifestations of BD, age at diagnosis, and therapy was collected from patients' medical records. BD patients were classified into two groups: mild to moderate disease and severe disease, according to the severity of disease manifestations, as suggested by Krause *et al.* (34). All disease-related variables were assessed regarding the presence of the *HLA-B*51* allele and its most frequent subtypes *HLA-B*51:01* and *HLA-B*51:08* in BD patients.

*Genotyping of HLA-B*51*

Genomic DNA was extracted from whole peripheral blood using the kit PureLink® Genomic DNA (Invitrogen™, Carlsbad, CA, USA). *HLA-B*51* DNA typing was performed in two steps. Firstly, samples were submitted to PCR with two sets of primers (Ambisolv™, One Lambda, Carlsbad, CA, USA) that addressed the *B*51-01* and the *B*51-24* alleles, respectively. It resulted in one 311 base pair (bp) fragment, and another 116 bp fragment, respectively, as shown in Supplementary Table S1 and Supplementary Figure S1. PCR products from positive samples for *HLA-B*51* alleles were then subjected to a second amplification procedure for high-resolution genotyping with the sequence-specific primers for class I locus B (PCR-SSP) (SeCore®Locus Sequencing kit, Invitrogen, Brown Deer, WI, USA). Sequencing products were submitted to capillary electrophoresis in 3130 Genetic Analyzer (Applied Biosystems), and raw sequencing data were assembled and analysed using uTYPE Software (Suppl. Fig. S2). Results were displayed as 4-digit alleles of the *HLA-B*51* for positive samples.

Statistical analysis

Continuous data were presented as mean and standard deviation or as the median and interquartile range (IQR) and categorical data as absolute and percentage figures. The Student's *t*-test or the Mann-Whitney's U-test were used for comparisons between two groups regarding continuous variables. The Chi-square test or Fisher's exact test was used to analyse categorical variables. Univariate and multivariate logistic regression models were performed to verify if *HLA-B*51* and its most frequent subtypes performed as susceptibility alleles for BD and to analyse independent associations with individual disease manifestations of BD. Results were expressed as odds ratio (OR) and 95% confidence interval (95CI). Significance level accepted was 5% ($p<0.05$). Statistical analysis was carried out with the IBM SPSS Statistics for Windows version 20.0 (Armonk, USA) and with GraphPad Prism version 6.00 for Windows (La Jolla, USA).

Table I. Distribution of *HLA-B*51* and its main subtypes according to demographic features in patients with Behçet's disease.

Variables	*HLA-B*51 status*		*p*
	*HLA-B*51* positive patients (n=25)	*HLA-B*51* negative patients (n=58)	
Caucasians, n (%)	10 (40.0)	14 (24.1)	0.144
Male patients, n (%)	11 (44.0)	26 (44.8)	0.945
Born in the Southeast region, n (%)	14 (56.0)	40 (71.4)	0.174
	*HLA-B*51:01* positive patients (n=15)	*HLA-B*51:01* negative patients (n=68)	
Caucasians, n (%)	7 (46.7)	17 (25.0)	0.119
Male patients, n (%)	7 (46.7)	30 (44.1)	0.857
Born in the Southeast region, n (%)	9 (60.0)	45 (68.2)	0.544
	*HLA-B*51:08* positive patients (n=5)	*HLA-B*51:08* negative patients (n=78)	
Caucasians, n (%)	1 (20.0)	23 (29.5)	1.000
Male patients, n (%)	3 (60.0)	34 (43.6)	0.652
Born in the Southeast region, n (%)	3 (60.0)	51 (67.1)	1.000

n: number of patients.

Results

Patients and healthy controls

Eighty-three BD patients and 258 HC were included in the study. BD patients were younger than HC [41.0 years (33.0–53.0) *vs*. 55.0 years (43.0–65.3); $p<0.0001$]. In contrast, BD patients and HC did not differ significantly regarding male gender proportion (44.6% *vs*. 38.4%; $p=0.315$) and percentage of Caucasians (28.9% *vs*. 39.1%; $p=0.125$). Most BD patients and HC included in this study were born in the Southeast region (65.1% and 67.4%) and in the Northeast region (32.5% and 26.7%) of Brazil.

Manifestations of Behçet's disease

The median time since the diagnosis of BD was 96.0 (48.0–168.0) months and most patients presented mucocutaneous manifestations of the disease (oral ulcers: 100%, genital ulcers: 80.7% and cutaneous involvement: 73.5%) followed by eye involvement (66.3%) (Supplementary Table S2). Among the 83 BD patients included in the study, 10 (12.0%) fulfilled only the ICBD criteria, and the remainder met both the ISG and ICBD criteria for BD. According to Krause's classification (29), at least one severe manifestation of BD was observed in 73 (88.0%) patients.

*The prevalence of HLA-B*51 and its main subtypes in Behçet's disease and controls*

The prevalence of *HLA-B*51* was higher in BD patients compared to HC (30.1% *vs*. 15.5%; $p=0.003$). Figure 1 depicts the frequency of each *HLA-B*51* subtype found in BD patients and HC. *HLA-B*51:01* was the most frequent *HLA-B*51* subtype in individuals who were positive for *HLA-B*51* as it was observed in 60.0% of BD patients and 77.5% of HC ($p=0.160$). In addition, *HLA-B*51:02*, *HLA-B*51:22,* and *HLA-B*51:29* were also found in both patients and HC. *HLA-B*51:08* was found only in BD patients, whereas *HLA-B*51:03, HLA-B*51:04, and HLA-B*51:55* were only found in HC.

*Distribution of HLA-B*51 and its subtypes according to demographic features*

In BD patients, the frequency of *HLA-B*51* and the subtypes *HLA-B*51:01* and *HLA-B*51:08* was similar between Caucasians and non-Caucasians, between male and female patients, and between patients born in the Southeast and the Northeast regions of Brazil (Table I).

*Distribution of HLA-B*51 and its main subtypes according to disease manifestations*

Table II describes comparisons between the presence of *HLA-B*51*, *HLA-B*51:01*, and *HLA-B*51:08* regarding disease manifestations in BD patients. The positivity of *HLA-B*51* was significantly lower in BD patients presenting neuro-Behçet compared to those without these manifestations ($p=0.034$). *HLA-B*51:01* was more frequent in BD patients presenting ocular involvement compared to patients without this manifestation ($p=0.014$), while no BD patient with vascular or neurologic manifestations presented *HLA-B*51:01* ($p=0.044$ and $p=0.020$, respectively). The frequency of *HLA-B*51:08* was significantly higher in BD patients presenting vascular manifestations compared to those without these manifestations ($p=0.012$).

The frequency of severe manifestations of BD was not different in patients presenting or not *HLA-B*51* (88.0% *vs*. 87.9%, $p=0.993$), *HLA-B*51:01* (93.3% *vs*. 86.8%, $p=0.479$) or *HLA-*

Table II. Distribution of *HLA-B*51* and its main subtypes according to disease manifestations in Behçet's disease.

Disease manifestations	*HLA-B*51 status*		*p*
	*HLA-B*51* positive patients (n=25)	*HLA-B*51* negative patients (n=58)	
Genital ulcers, n (%)	23 (92.0)	44 (75.9)	0.087
Skin involvement, n (%)	21 (84.0)	40 (69.0)	0.155
Joint complaints, n (%)	11 (44.0)	19 (32.8)	0.328
Ocular inflammation, n (%)	20 (80.0)	35 (60.3)	0.082
Vasculo-Behçet, n (%)	3 (12.0)	12 (20.7)	0.345
Neuro-Behçet, n (%)	2 (8.0)	17 (29.3)	0.034*
	*HLA-B*51:01* positive patients (n=15)	*HLA-B*51:01* negative patients (n=68)	
Genital ulcers, n (%)	14 (93.3)	53 (77.9)	0.171
Skin involvement, n (%)	12 (80.0)	49 (72.1)	0.528
Joint complaints, n (%)	8 (53.3)	22 (32.4)	0.126
Ocular inflammation, n (%)	14 (93.3)	41 (60.3)	0.014*
Vasculo-Behçet, n (%)	0 (0.0)	15 (22.1)	0.044*
Neuro-Behçet, n (%)	0 (0.0)	19 (27.9)	0.020*
	*HLA-B*51:08* positive patients (n=5)	*HLA-B*51:08* negative patients (n=78)	
Genital ulcers, n (%)	4 (80.0)	63 (80.8)	0.966
Skin involvement, n (%)	4 (80.0)	57 (73.1)	0.734
Joint complaints, n (%)	1 (20.0)	29 (37.2)	0.438
Ocular inflammation, n (%)	3 (60.0)	52 (66.7)	0.760
Vasculo-Behçet, n (%)	3 (60.0)	12 (15.4)	0.012*
Neuro-Behçet, n (%)	0 (0.0)	19 (24.4)	0.209

n: number of patients; the asterisk flags significant associations.

Table III. Multivariate analysis to evaluate the association of demographic, clinical, and immunogenetic factors with *HLA-B*51* as a risk factor for Behçet's disease.

Variables	Odds ratio	95% confidence interval	*p*
Female sex	0.707	0.419–1.193	0.194
Caucasian	1.545	0.880–2.711	0.130
Birth in the Southeast region	0.872	0.495–1.536	0.635
*HLA-B*51*	2.410	1.332–4.361	0.004*

The asterisk flags significant associations.

*B*51:08* (80.0% *vs*. 88.5%, *p*=0.573), respectively. Moreover, age at diagnosis of BD was not different in those presenting or not *HLA-B*51* (35.7±13.0 years *vs*. 32.1±11.1 years, *p*=0.216), *HLA-B*51:01* (34.9±12.4 years *vs*. 32.8±11.7 years, *p*=0.541) or *HLA-B*51:08* (36.4±18.0 years *vs*. 33.0±11.4 years, *p*=0.538), respectively.

*Multivariate analysis for the clinical associations of HLA-B*51 and its main subtypes*

Based on the associations found in the comparisons of the frequency of *HLA-B*51*, *HLA-B*51:01* and *HLA-B*51:08* with disease features of BD, multivariate models of logistic regression were built to analyse predictors for the development of BD and severe disease manifestations such as neuro-Behçet, ocular involvement, and vasculo-Behçet. In the multivariate model that included BD patients and HC, the presence of the *HLA-B*51* allele was independently associated with BD (Table III). In the evaluation of predictors of neuro-Behçet, no significant association was found with *HLA-B*51* carriage. Still, the birth in the Southeast region of Brazil and ocular involvement were inversely associated with neuro-Behçet (Table IV). On the other hand, the presence of neuro-Behçet was independently associated with a lower risk for ocular involvement in BD. In contrast, no significant association could be found between *HLA-B*51:01* and eye inflammation (Table IV). The presence of *HLA-B*51:08* was an independent predictor for the development of the vasculo-Behçet (Table IV).

Discussion

To the best of our knowledge, it is the first time that the prevalence and clinical-demographic associations of *HLA-B*51* and its subtypes are addressed in Brazilian BD patients. In this study, BD was associated with a higher frequency of *HLA-B*51* compared to HC, and this allele was an independent risk factor for the development of BD in the analysed cohort. *HLA-B*51:01* was the most frequent subtype found in BD and HC, while *HLA-B*51:08* was found only in BD patients and not observed in HC. No independent association was found between *HLA-B*51* or its subtype *HLA-B*51:01* and clinical manifestations of BD. However, *HLA-B*51:08* was an independent predictor of vascular involvement in BD.

The prevalence of *HLA-B*51* in Brazilian BD patients (30.1%) was lower than the pooled prevalence of 55.0-63.5% reported for *HLA-B*5/B*51* in BD patients from countries in East Asia, Middle East, North Africa and Southern Europe (28), but it is similar to the prevalence of *HLA-B*51* (25-36%) reported in Caucasian patients with BD from Ireland and the United Kingdom (35, 36). The prevalence of *HLA-B*51* in Brazilian BD patients is slightly lower than that reported in BD patients from Portugal (41%), and this may be related to the much more varied genetic background in the Brazilian as compared to the Portuguese population (37). Although the prevalence of *HLA-B*51* in Brazilian BD patients was lower than that observed in other population groups, *HLA-B*51* was still an independent risk factor for BD in our country. Nonetheless, the 15% prevalence of *HLA-B*51* found in Brazilian controls falls within the range reported for the pooled prevalence (11.2-

Table IV. Multivariate analysis to evaluate associations of demographic, clinical, and immunogenetic factors with the development of severe manifestations of Behçet's disease.

Variables	Odds ratio	95% confidence interval	*p*
Independent variables and the risk to develop neurologic manifestations			
Female sex	2.200	0.310 – 15.606	0.430
Caucasian	0.418	0.066 – 2.642	0.354
Birth in the Southeast region	0.055	0.008 – 0.394	0.004*
Genital ulcers	0.536	0.044 – 6.546	0.625
Cutaneous involvement	3.496	0.514 – 23.804	0.201
Joint complaints	1.181	0.265 – 5.273	0.827
Ocular involvement	0.053	0.009 – 0.313	0.001*
Vasculo-Behçet	1.055	0.147 – 7.561	0.958
*HLA-B*51*	0.119	0.014 – 1.036	0.054
Independent variables and the risk to develop ocular involvement			
Female sex	0.859	0.249 – 2.971	0.811
Caucasian	0.829	0.215 – 3.200	0.786
Birth in the Southeast region	0.212	0.041 – 1.094	0.064
Genital ulcers	0.160	0.023 – 1.091	0.061
Cutaneous involvement	2.248	0.590 – 8.568	0.235
Joint complaints	1.296	0.372 – 4.517	0.685
Neuro-Behçet	0.068	0.012 – 0.392	0.003*
Vasculo-Behçet	2.192	0.452 – 10.640	0.330
*HLA-B*51:01*	6.231	0.673 – 57.649	0.107
*Independent variables and the risk to develop v*ascular involvement			
Female sex	0.759	0.159 – 3.627	0.730
Caucasian	2.539	0.365 – 17.660	0.346
Birth in the Southeast region	3.555	0.602 – 20.991	0.162
Genital ulcers	0.413	0.067 – 2.525	0.338
Cutaneous involvement	1.293	0.251 – 6.671	0.759
Joint complaints	0.747	0.157 – 3.558	0.714
Ocular involvement	2.339	0.377 – 14.524	0.362
Neuro-Behçet	1.956	0.230 – 16.606	0.539
*HLA-B*51:08*	14.843	1.550 – 142.115	0.019*

The asterisk flags significant associations.

21.7%) of *HLA-B*51* in controls from East Asia, Middle East, North Africa, Northern and Southern/Eastern Europe (28).

Some studies have assessed the role of *HLA-B*51* subtypes as risk factors for BD in different populations. In line with our findings, *HLA-B*51:01* was the most frequent subtype associated with BD in this cohort and was followed by *HLA-B*51:08* (38-49). In our study, despite the high frequency of *HLA-B*51:01* in BD patients, it was also the most frequent subtype observed in HC. This same finding has been reported in studies performed in different countries such as Greece, Italy, Israel, Spain, Germany, Japan, Saudi Arabia, and in Middle Eastern patients with BD where the majority of individuals from the control group presented *HLA-B*51:01* (38, 40-44, 46-50). Conversely, the prevalence of *HLA-B*51:01* in Turkey was lower than that previously reported by other studies in both groups, BD and controls (*i.e.* 45.5% and 40.0%, respectively). However, it was in accordance with the results from *HLA-B*51* subtypes in Brazilian BD patients and HC, since different *HLA-B*51* subtypes were also found at a low frequency in patients and controls from Turkey (39, 51).

Despite the low prevalence of the subtype *HLA-B*51:08* in BD patients, it seems to be strongly associated with BD as we could not find this allele in any HC. Some studies have shown similar findings regarding *HLA-B*51:08, i.e.*, a relatively low prevalence in BD patients and its absence in control groups (40, 41, 47). On the other hand, four studies performed in Turkey, Spain, Middle Eastern descents, and in Germany have described a low frequency of *HLA-B*51:08* in BD patients and controls (39, 48-50).

In this study, some associations were observed of *HLA-B*51* and its main subtypes with some clinical manifestations of BD. The prevalence of *HLA-B*51* was significantly lower in BD patients presenting neuro-Behçet, whereas *HLA-B*51:01* was more frequently found in patients presenting ocular inflammation. *HLA-B*51:01* was not found in BD patients with neuro-Behçet or with vascular involvement. Despite these significant associations, no independent association was observed between *HLA-B*51* or *HLA-B*51:01* and isolated clinical manifestations of BD in multivariate analysis. The inverse association between ocular inflammation and neuro-Behçet and the behaviour of birth in the Northeast of Brazil as a risk factor for the development of neuro-Behçet are intriguing findings that deserve further investigation in future studies.

Previous studies have analysed associations between *HLA-B*5/B*51* carriage and disease manifestations in BD patients, with controversial results even though some of these studies had large sample sizes (52-58). Associations between *HLA-B*5/B*51* and poor prognosis of BD with ocular involvement or neuro-Behçet have been described (54, 57-59), but these findings have not been confirmed in other studies (53, 55, 56). The association between *HLA-B*5/B*51* with thrombophlebitis and skin involvement of BD have also been reported (53, 55, 56). One study could not find any association between *HLA-B*5* and isolated clinical manifestations of BD (52). A meta-analysis that included 72 studies performed in 74 different population groups found that the *HLA-B*5/B*51* carriage was common in male BD patients and it was moderately associated with a higher prevalence of genital ulcers, skin and eye manifestations but not with severe disease manifestations such ocular inflammation, neuro-Behçet and vascular involvement (28).

A few studies have assessed associations between *HLA-B*51* subtypes and isolated manifestations of BD (38,48,51). *HLA-B*51:01* was significantly associated with erythema nodosum and with an earlier age at disease onset in BD patients from Greece

(38). No association regarding *HLA-B*51:01* carriage was found with isolated BD manifestations and with ocular involvement in BD patients from Turkey and Middle Eastern, respectively (48, 51).

This is the first demonstration that *HLA-B*51:08* is independently associated with vascular manifestations in BD. Only one previous study had analysed associations between ocular involvement in BD and *HLA-B*51:08*, but no association was observed between *HLA-B*51:08* carriage and this disease manifestation (48).

Two major limitations of this study include the relatively small sample size of the BD group as an impairment for subgroup analysis and the inclusion of 10 BD patients fulfilling only the ICBD criteria since these criteria have low specificity in non-endemic areas. Another limitation of this study is its hospital-based nature, since it may be a potential source of referral bias with the inclusion of a high proportion of severe BD patients in the study. One minor limitation of this study was to focus only on *HLA-B*51* and its subtypes by HLA high resolution genotyping in BD. Naturally, this approach restricts the possibilities in uncovering possible associations between BD and other HLA-susceptibility genes located at MHC-class I locus A and C or even with alleles at the MHC-class II loci.

Conclusions

*HLA-B*51* was more frequent in Brazilian BD patients compared to HC, and it presented characteristics of an independent risk factor for BD. The subtypes *HLA-B*51:01* and *HLA-B*51:08* were the most frequent amongst BD patients, but *HLA-B*51:01* was observed at a high frequency also in HC, while *HLA-B*51:08* was only found in BD patients. The *HLA-B*51:08* subtype was independently associated with vascular manifestations of BD.

Acknowledgments

The authors thank Gisele Fabianne Rampim from Instituto de Imunogenética (IGEN) for the technical support in analysing data from HLA sequencing.

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