Genetics of Behçet's disease in Sardinia: two distinct extended HLA haplotypes harbour the B*51 allele in the normal population and in patients

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Received on April 18, 2012; accepted in revised form on August 29, 2012.

Clin Exp Rheumatol 2012; 30 (Suppl. 72): S51-S56.

Key words: Behçet's disease, genetics, immunogenetics, HLA, HLA-B51, TNF-alpha

Funding: this work was partially supported by the Fondazione Pasteur-Cenci Bolognetti.

Competing interests: none declared.

ABSTRACT

Objective. *To define the contribution of HLA genes and extended HLA haplotypes to the susceptibility to Behçet's disease (BD) in Sardinia.*

Methods. *Forty-five unrelated Sardinian patients with BD, diagnosed according to the ISG criteria, 45 HLA-B*51 positive and 185 unselected healthy controls were enrolled in the study. DNA samples were typed for HLA class I and class II alleles and genotyped for microsatellites (MICA-TM) and single-nucleotide polymorphisms (rs1264457 HLA–E; rs2281820 motilin; rs1799724 at -857, rs361525 at -238 TNF-alpha) spanning the HLA region.*

Results. *HLA-B*5101 was confirmed as conferring susceptibility to BD (p_c=0.0042; OR=4.4; 95% CI=2.0 to 9.6). It is noteworthy that in Sardinia this allele was found more frequently within a haplotype (HLA-A2; -Cw2; -B*5101; -DRB1*11; -DQA1*05; -DQB1*03) that reached its highest frequency in patients with BD. Linkage disequilibrium analysis showed the existence of an additional B*51 haplotype (HLA-A2; -Cw2; -B*5101; -DRB1*04; -DQA1*03; -DQB1*03) not associated with susceptibility to the disease.*

Conclusion. *In Sardinia, the BD-associated HLA-B*5101 allele is inherited as part of two distinctive haplotypes differently distributed in patients and controls. These findings can be interpreted as suggestive of the presence of additional genes within the MHC region conferring susceptibility to BD. The hypothesis that an environmental pressure could have contributed to the preservation of the BD-associated HLA haplotype in Sardinia is also discussed.*

Introduction

Behçet's disease (BD) is a multisystem vasculitis characterised by an immune-mediated pathogenesis which is suspected to be triggered by environmental factors in subjects harbouring a genetically favourable substrate (1).

The association of HLA-B*51 with BD is the major evidence of a genetic susceptibility to the disease and partly explains its geographical distribution, bordered between latitudes 30° N and 45° N spanning from the Mediterranean basin to the Far East, which confers to it the name of the Silk Road disease (2). It has been computed that the HLA-B*51 accounts for 32–52% of BD cases within various ethnic subgroups (3). However, HLA-B*51 is not the only genetic factor contributing to BD (4). Candidate gene analysis, microsatellite polymorphisms and genome-wide association studies have suggested the existence of additional genes, lying within and outside the MHC region, implicated in BD susceptibility and pathogenesis (5, 6). The HLA-B*5101 allele, together with the closely-linked MHC class I chain-related gene A (MICA) and tumour necrosis factor-α (TNF-α), appear to be the main MHC-related factors associated with BD. Other genes, such as the non-classical HLA-E and HLA-G, have also been reported to be involved in BD pathogenesis. However, it is difficult to single out the exact role of each gene because of the strong Linkage Disequilibrium (LD) which characterises the HLA region, and it cannot be excluded that more than one gene in the same haplotype cooperate to confer susceptibility to BD (7).

The haplotypic distribution of HLA in Sardinia, compared to other Mediterranean populations, is characterised by a very high number of rare haplotypes and by a small number of frequent haplotypes (8, 9). The Sardinian population represents an ancient genetic isolate

with a highly preserved allelic and haplotypic distribution of genes originated from a relatively large group of founders, fixed during thousands years as a consequence of geographic isolation, endogamy, genetic drift and exposure to endemic infection with microbial-driven selection. *Plasmodium Falciparum*, a highly lethal infectious agent endemic in Sardinia until its eradication 60 years ago, has been reported to exert a selective pressure among inhabitants of infested area with an effective selection on HLA alleles (10).

The aim of this study was to investigate the association of HLA genes and extended haplotypes with susceptibility to BD in Sardinian patients.

Patients and methods

Patients and controls

Forty-five unrelated consecutive Sardinian patients with BD diagnosed according to the ISG criteria, 39 referring to the Rheumatology Unit of Cagliari and 6 to the Rheumatology Unit of Sassari, were enrolled in the study. Forty-five consecutive HLA-B*51 positive (B*51-HC) and 185 unselected (HC) healthy bone marrow donors served as controls. All patients and controls came from various areas of Sardinia and were representative of the population distribution. Since inhabitants from the municipality of Alghero and the islands of La Maddalena and San Pietro are of a recent non-Sardinian origin, they were excluded from the control population. Both patients and controls gave their informed consent to the study which has been approved by the local ethics committee (n. 224/CE) and performed according to the principles of the Good Clinical Practice and Declaration of Helsinki.

Genotyping

All patients and controls were typed for HLA-A, B, C, DRB1, DQA1 and DQB1 using commercial kits (HLA SSP kits; Biotest, Dreieich, Germany). High resolution subtyping was applied to all HLA-B*51 positive patients and B*51-HC using a PCR-SSP kit (Olerup SSP AB, Saltsjobaden, Sweden). All patients and HC controls were typed for MICA transmembrane microsatellite polymorphisms (short tandem repeat [STR] MICA) and for the following single-nucleotide polymorphisms (SNPs): rs1264457, mapping in the third exon of the HLA–E gene; rs2281820 in the motilin gene; rs1799724 at -857 and rs361525 at -238 of the tumour necrosis factor alpha (TNF-α) promoter gene.

MICA microsatellite polymorphisms were determined as previously described by heteroduplex analysis on genomic DNA amplified by PCR (11). The rs1264457, rs1799724, rs361525 and rs2281820 frequencies were determined using the Taqman SNP genotyping assay (Applied Biosystems, Carlsbad, California, USA) according to the manufacturer's instructions.

Statistical analysis

To assess the differences in the proportions of polymorphic alleles and disease associations in HC *versus* BD patients, chi-square test or two-tailed Fisher's exact test were performed using MedCalc software (version 9.5.0.0, Mariakerke, Belgium). The strength of association was estimated by calculating the odds ratio (OR) with 95% Confidence Interval (95% CI). Under the assumption of independence, a value of $p<0.05$ was considered statistically significant where Bonferroni correction was applied for multiple comparisons to all novel associations, with a correction factor derived from the number of alleles found; p_c indicates where the Bonferroni correction was applied.

The LD between pairs of loci was calculated performing a likelihood-ratio test, whose empirical distribution is obtained by a permutation procedure using the statistical software Arlequin (version 3.11, Bern, Switzerland). The significance ($p<0.01$) of the observed likelihood ratio is found by computing the null distribution of this ratio under the hypothesis of LD, using a permutation procedure. All markers were in Hardy-Weinberg equilibrium. Maximum-likelihood haplotype frequencies and their distribution were estimated using an Expectation-Maximisation algorithm and polymorphic alleles within each investigated HLA locus were constructed into extended haplotypes.

Results

Allele distribution

The phenotype frequency (*pf*) of the HLA-B*51 allele was 42.2% in patients, significantly higher than that of HC group (14.2%) (p_c=0.0042; OR=4.4; 95% CI=2.0 to 9.6). No other alleles were observed as independently associated with BD. The frequencies of investigated alleles are reported in Table I.

All B*51 positive BD carried the B*5101 allele (n=18) but one who carried the B*5108 (n=1). All B*51 positive HC were B*5101 (n=45).

Linkage disequilibrium analysis

The LD analysis of HLA–A, B, C, DR and DQ loci highlighted the presence of 5 and 9 loci in LD ($p<0.01$) among B*51-BD and B*51-HC respectively (Fig. 1). HLA-A2 and -Cw2 alleles were highly represented in B*51-HC (77.8% and 40.0%, respectively) and B*51-BD (68.4% and 31.6%, respectively) when compared to random HC (43.3% and 8.3%, respectively). The DRB1*11 and the DQA1*05:05/B1*03 dimer were more frequent in B*51-BD (52.6% and 63.2%, respectively) than in random HC (29.2% and 25.0%, respectively) and B*51-HC (48.8% and 37.7%, respectively) (Table II). DRB1*04 and the DQA1*03:01/B1*03 dimer followed an opposite trend being highly represented in the B*51-HC (48.8% and 42.2%, respectively) when compared to random HC (27.5% and 21.7%, respectively) and B*51-BD (21.1% and 10.5%, respectively). In B*51-BD the B locus was in high LD with the DR locus whereas the frequency of DQA1/DQB1 alleles is increased as an effect of the strong LD with DR locus (Fig. 1). Conversely, in the HLA-B51 positive HC, the B locus was in high LD with all the other investigated loci, suggesting the existence of a highly preserved haplotype among controls. Based on these data the HLA-B*5101 allele seems to be inherited as part of different haplotypes in HC and BD (Fig. 2). It is noteworthy that none of the investigated SNPs showed a different distribution in the two HLA-B51 positive cohorts.

*HLA-B*51 geographic distribution*

The *pf* of B*51 resulted significantly

Table I. Phenotype frequencies of HLA-A, -B, -Cw, -DR, -DQA1, DQB1, MICA alleles and genotype frequencies of HLA-E, TNF-α-857, TNF-α-238 and motilin alleles in Sardinian healthy controls and Behçet's disease patients.

Marker (n. of healthycontrols/ n. of BD patients)	Healthy controls n. (%)	BD patients n. (%)	*p*	p_{corr}
HLA-A (120/45)				
A*1	13 (10.8)	2 (4.4)	ns	
A*2	60 (50.0)	27 (60.0)	ns	
A*3	13 (10.8)	1 (2.2)	ns	
A*11	18 (15.0)	9 (20.0)	ns	
A*24	23 (19.2)	10 (22.2)	ns	
A*26	6 (5.0)	4 (8.9)	ns	
A*29	4 (3.3)	1 (2.2)	ns	
A*30	42 (35.0)	17 (37.8)	ns	
A*32	23 (19.2)	5 (11.1)	ns	
A*33	10 (8.3)	3 (6.7)	ns	
A*68	9 (7.5)	3 (6.7)	ns	
A*69	4 (3.3)	1 (2.2)	ns	
A*74	2 (1.7)	1 (2.2)	ns	
HLA-E (185/40)				
A/A	48 (26.0)	10 (25.0)	ns	
A/G	100 (54.0)	26 (65.0)	ns	
G/G	37 (20.0)	4 (10.0)	ns	
HLA-Cw (120/45)				
Cw1	12 (10.0)	8 (17.8)	ns	
Cw2	12 (10.0)	10 (22.2)	ns	
Cw3	6 (5.0)	2 (4.4)	ns	
Cw4	38 (31.7)	11 (24.4)	ns	
Cw5	40 (33.3)	16 (35.6)	ns	
Cw6	21 (17.5)	7 (15.6)	ns	
Cw7	46 (38.3)	10 (22.2)	ns	
Cw8	16 (13.3)	2 (4.4)	ns	
Cw12	18 (15.0)	9 (20.0)	ns	
Cw14	2 (1.7)	1 (2.2)	ns	
Cw15	10 (8.3)	3 (6.7)	ns	
HLA-B (120/45)				
B*8	7 (5.8)	1 (2.2)	ns	
B*13	4 (3.3)	3 (6.7)	ns	
B*14	18 (15.0)	2 (4.4)	ns	
B*15	4 (3.3)	1 (2.2)	ns	
B*17	0 (0.0)	1 (2.2)	ns	
B*18	54 (45.0)	19 (42.2)	ns	
B*27	8 (6.7)	3 (6.7)	ns	
B*35	36 (30.0)	10 (22.2)	ns	
B*37	5 (4.2)	2 (4.4)	ns	
B*38	4 (3.3)	2 (4.4)	ns	
B*39	2 (1.7)	1 (2.2)	ns	
B*40	2 (1.7)	1 (2.2)	ns	
B*44	8 (6.7)	3 (6.7)	ns	
B*49	8 (6.7)	1 (2.2)	ns	
B*50	4 (3.3)	2 (4.4)	ns	
B*51	17 (14.2)	19 (42.2)	*0.0002*	*0.0042*
B*53	1 (0.8)	1 (2.2)	ns	
B*55	3 (2.5)	1 (2.2)	ns	
B*56	10 (8.3)	3 (6.7)	ns	
B*57	7 (5.8)	2 (4.4)	ns	
B*58	16 (13.3)	6 (13.3)	ns	
MICA (138/39)				
A4	79 (57.2)	17 (43.6)	ns	
A5	19 (13.8)	5 (12.8)	ns	
A5.1	30 (21.7)	10 (25.6)	ns	
A6	59 (42.8)	22 (56.4)	ns	
A9	51 (37.0)	16 (41.0)	ns	
TNF-857 (185/41)				
C/C	131 (70.8)	28 (68.3)	ns	
C/T	43 (23.2)	13 (31.7)	ns	
T/T	11 (5.9)	0 (0.0)	ns	
TNF-238 (185/42)				
G/G	132 (71.4)	29 (69.0)	ns	
G/A	43 (23.2)	11 (26.3)	ns	
A/A	10 (5.4)	2 (4.7)	ns	
HLA-DR (120/45)				
DRB1*01	23 (19.2)	4 (9.3)	ns	
DRB1*03	48 (40.0)	18 (41.9)	ns	
DRB1*04	33 (27.5)	11 (25.6)	ns	
DRB1*07	13 (10.8)	4 (9.3)	ns	
DRB1*10	10 (8.3)	3 (7.0)	ns	
DRB1*11	35 (29.2)	16 (37.2)	ns	
DRB1*12	4 (3.3)	1 (2.3)	ns	
DRB1*13	6 (5.0)	5 (11.6)	ns	
DRB1*14	5 (4.2)	1 (2.3)	ns	
DRB1*15	7 (5.8)	1 (2.3)	ns	
DRB1*16	39 (32.5)	14 (32.6)	ns	
HLA-DQA1(120/36)				
DQA1*01:01	22 (18.3)	5 (13.9)	ns	
DQA1*01:02	45 (37.5)	13 (36.1)	ns	
DQA1*01:03	4 (3.3)	2 (5.6)	ns	
DQA1*01:04	5 (4.2)	1 (2.8)	ns	
DQA1*01:05	10 (8.3)	2 (5.6)	ns	
DQA1*02:01	13 (10.8)	4 (11.1)	ns	
DQA1*03:01	23 (19.2)	6 (16.7)	ns	
DQA1*03:03	5 (4.2)	2 (5.6)	ns	
DQA1*04:01	1 (0.8)	0 (0)	ns	
DQA1*05:01	48 (40.0)	13 (36.1)	ns	
DQA1*05:05	42 (35.0)	16 (44.4)	ns	
HLA-DQB1(120/42)				
DQB1*02	54 (45.0)	19 (45.2)	ns	
DQB1*03	67 (55.8)	25 (59.5)	ns	
DQB1*04	1 (0.8)	0 (0.0)	ns	
DQB1*05	69 (57.5)	19 (45.2)	ns	
DQB1*06	10 (8.3)	7 (16.7)	ns	
MOTILIN (104/40)				
C/C	41 (39.4)	21 (53.8)	ns	
C/T	46 (44.2)	17 (43.6)	ns	
T/T	17 (16.3)	3 (7.7)	ns	

higher (p=0.02; OR=7.0; 95% CI=1.2 to 38.9) in patients native of the central-eastern Sardinian district (77.8% *vs*. 20% in general population), which is physically bordered by the island's highest mountains and by the most important river, than in patients inhabiting the other districts (33.3%; 9% in the general population), characterised by lowlands, reflecting the geographic distribution of this allele in healthy controls (8).

Discussion

To our knowledge, this is the first report on genetic susceptibility to BD in Sardinia. The strength of this study is that cases and controls were carefully matched by genetic ancestry to

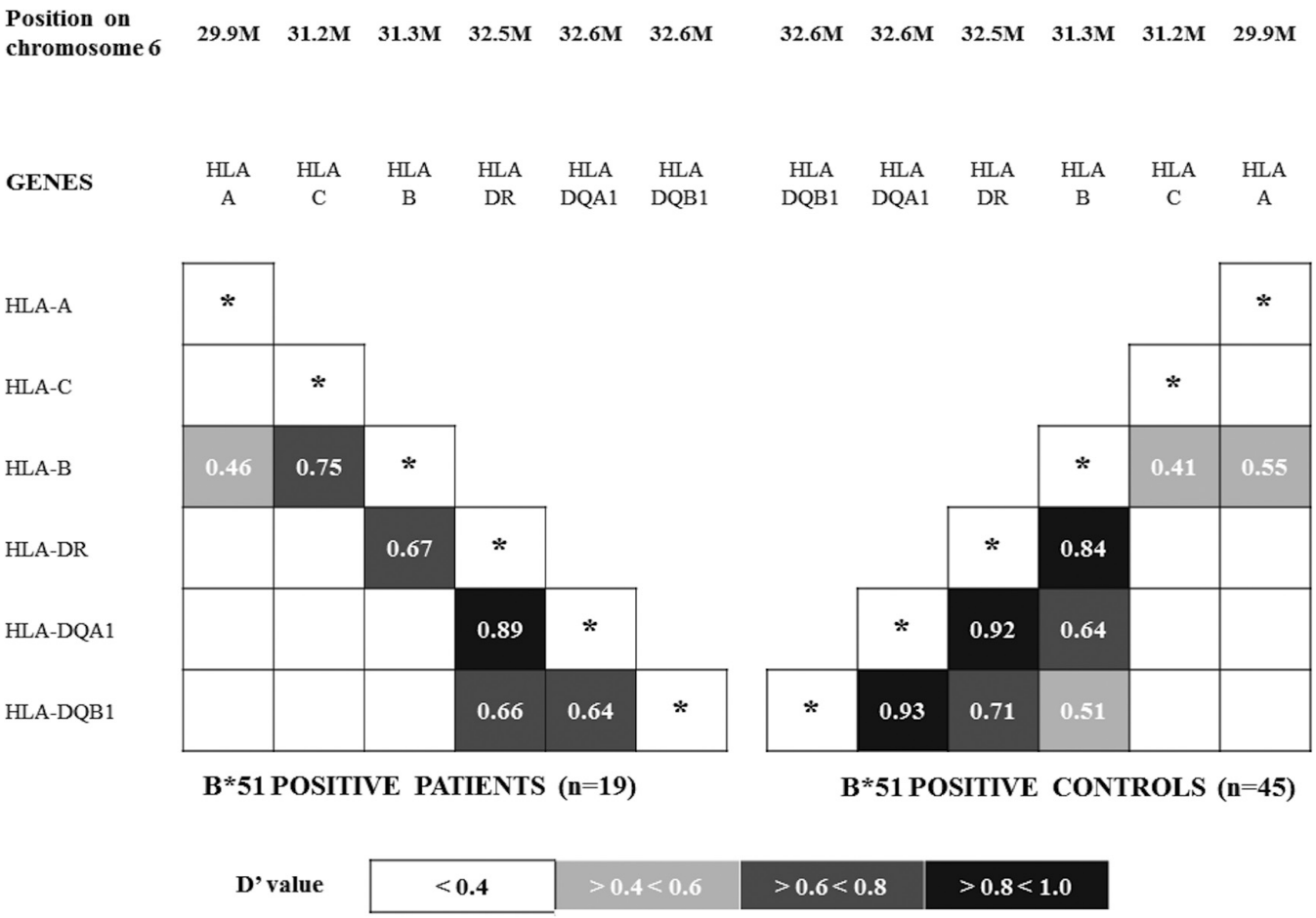

Fig. 1. Significance of linkage disequilibrium (LD) between pairs of HLA loci. Significance for LD were calculated performing a likelihood-ratio test using Arlequin software; LD levels are expressed as D' value for $p \leq 0.01$. Approximate locations on chromosome 6 are indicated.

Table II. Relative frequencies of the most highly represented HLA-A. C. DR alleles and DQA1/B1 eterodimers of the HLA-B51 haplotypes present in Sardinian Behçet's disease (BD) patients and healthy controls.

Marker (random healthy controls/ B*51+ healthy controls/ B*51+ BD patients)	Allele Frequency in general Sardinian population, %	random healthy controls n. (%)	B*51+ healthy controls n. (%)	B*51+ BD patients n. (%)	*p* (B*51+ HC *vs.* random HC)	OR (95% CI)	*p* (B*51+ BD *vs.* random HC)	OR (95% CI)	*p* (B*51+ BD *vs.* B*51+ HC)	*OR* (95% CI)
HLA-A (120/45/19)										
A*2	29.2	52 (43.3)	35 (77.8)	13 (68.4)	<0.001	4.5 (2.0-10.0)	0.04	2.8 (1.0-7.9)	ns	
A*32	10.0	23 (19.2)	4 (8.8)	4 (21.1)	ns		ns		ns	
HLA-Cw (120/45/19)										
Cw2	5.0	10 (8.3)	18 (40.0)	6 (31.6)	<0.001	7.3 (3.0-17.6)	0.006	5.0 (1.6-16.2)	ns	
Cw7	21.7	46 (38.3)	16 (35.5)	5 (26.3)	ns		ns		ns	
HLA-DR (120/45/19)										
DR*4	14.8	33 (27.5)	22 (48.8)	4 (21.1)	0.01	2.5 (1.2-5.1)	ns		0.04	0.3 (0.1-0.9)
DR*11	15.7	35 (29.2)	22 (48.8)	10 (52.6)	0.02	2.3 (1.1-4.6)	0.04	2.6 (1.0- 7.2)	ns	
DR*13	2.6	6 (5.0)	3 (6.6)	2 (10.5)	ns		ns		ns	
DQA1/B1 (120/45/19)										
DQA1*05:05/B1*03	13.5	30 (25.0)	17 (37.7)	12 (63.2)	ns		0.001	5.1 (1.8-14.2)	ns	
DQA1*03:01/B1*03	11.4	26 (21.7)	19 (42.2)	2 (10.5)	0.01	2.6 (1.2-5.5)	ns		0.02	0.1 (0.0-0.7)

avoid systematic bias due to population stratification. Although we have been able to recruit only a relatively small number of patients, due to both the low frequency of BD and the size of the general population in Sardinia (about 1.6 million people), our cohort accounts for an estimated prevalence of 3/100.000, similar to that reported in peninsular Italy (2.5-3.8/100.000) (12). However, as sample size is a major issue in many genetic studies, with both

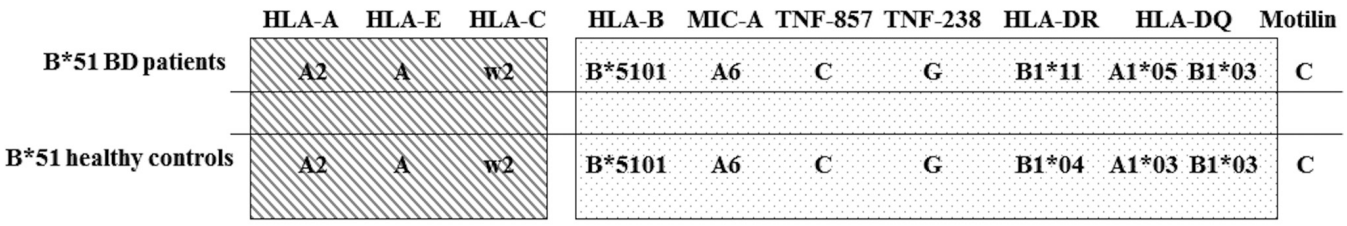

Fig. 2. Schematic representation of the HLA-B*51 haplotypes in Behçet's disease patients and healthy controls. The haplotypes shown in the dotted area represent >40% of all possible HLA-B*51 haplotypes in patients and healthy controls respectively. When considered together from locus A to locus DQ*B1 the haplotypes shown represent <10% and <20% of all possible HLA-B*51 haplotypes in patients and healthy controls, respectively.

type 1 and 2 errors being more likely to occur in smaller study populations, caution is advised when interpreting our results so long as the foregoing discrepancy may be related to the size of the population under study.

Our results confirmed the previous described association between HLA-B51 and BD. Moreover, we identified two extended haplotypes: the A2-Cw2-B*5101-DRB1*11-DQA1*05-DQB1*03, which marks the B*51 patients with BD in Sardinia, and the A2-Cw2-B*5101-DRB1*04-DQA1*03-DQB1*03, which is significantly more frequent in Sardinian HC than in BD.

The other markers investigated are not different between patients and controls suggesting that the increased frequency of these alleles in the HLA-B51 positive subjects is due to LD. It is noteworthy that the two haplotypes differ in the HLA-class II region. Previous studies in Italian and Turkish BD patients showed the positive association between B5/51 and DR5 (13, 14) and DR5-DQw3 (14). These serological markers correspond to the molecularly defined DRB1*11 and DQA1*05-DQB1*03 alleles, respectively. Therefore, the B-DR-DQ haplotype conferring susceptibility to BD in Sardinian patients did not differ from that of other Mediterranean populations. On the other hand, the haplotype B*51-DRB1*04-DQA1*03-DQB1*03 was not pinpointed in these studies and, interestingly, is significantly more frequent in the B*51 healthy controls, suggesting that it lacks a relevant predisposing factor to BD. This finding is worth mentioning also because its frequency (18%) and LD (χ^2=516.8; Δ=61.8) is higher among the B*51 islanders and is part of the small number of the preserved ancestral haplotypes in the Sardinian population (8, 15).

Since almost all HLA-B51-positive subjects carry the susceptible HLA-B*5101 allele, the distinct haplotypes distribution between patients and healthy controls and the reason for susceptibility to BD must not be ascribed to the harbouring of different B*51 sub-alleles. Contrarily, since HLA loci in the Sardinian population are clustered in a number of defined ancestral haplotypes, which are highly preserved mostly between the HLA-B and -DR loci, it might be speculated that a susceptibility or co-susceptibility factor, acting independently from or together with HLA-B*51, lies within this 1.000kb portion of the MHC region, which harbours the so-called HLA class III region including the TNF-α gene promoter. Data obtained from several surveys suggested that genes belonging to the HLA class III region (MICA, TNF-α polymorphisms) may play a role in BD susceptibility, independently or as part of extended haplotypes defined by LD with susceptibility genes (7). Particularly, the -1031C and -238A TNF-α promoter polymorphisms have been described as associated with an increased risk whereas the -857T with a decreased risk of BD (16). Although we did not find any suggestion for this in our population it might be still possible that TNF-α plays a pivotal role in BD pathogenesis

As regards the results on distinctive geographical distribution of HLA-B51 in Sardinia, it should be highlighted that this allele shows an overall frequency of 7%, which is the lowest among the populations in the Mediterranean area (17). Since it has been proved that Sardinians do not show genetic characteristics belonging to invader populations, the islander genetic structure finds support in the consequences of some evolutionary factors that acted over time, such as founder effect, isolation, microbial-driven selection and bottleneck effect (18).

Since it is likely that common susceptibility genes for immune-mediated diseases are selected in populations by their additional ability to confer resistance or susceptibility to infectious diseases, as recently supposed in ankylosing spondylitis (19, 20), asthma (21), type 1 diabetes (22) and multiple sclerosis (23-25), it might be speculated the same evolutionary model for BD. Contu *et al*. demonstrated that HLA-B51 frequency has a negative correlation with past malaria incidence, suggesting an adaptive phenomenon among islanders inhabitants in Sardinia (26). TNF-α polymorphism distribution has been addressed as the result of selective pressure on TNF-α itself or on neighbouring genes, including the HLA, exerted by infectious disease with a high burden of mortality among young people such as malaria by *Plasmodium Falciparum* or plague by *Yersinia Pestis* (25, 27). Such a possible explanation to the distinctive distribution of BD susceptibility genes and consequently to the geoepidemiology of BD deserves further investigation.

In conclusion, the HLA-B*5101 was the only susceptibility factor for BD, among those investigated in the Sardinian population. However, it appeared inherited as part of two distinctive haplotypes, with different distribution between patient and control cohorts, suggesting the existence of a susceptibility or co-susceptibility gene(s). Although a founder effect might be considered, such distinctive haplotypic distribution and disease susceptibility may result from one, or the superimposition of more, pathogen-driven evolutionary effect,

determining natural selection acting as predisposing to the development of immune-mediated inflammatory diseases. Future studies need to be addressed to clarify the evolution of susceptibility to BD and might shed some light on the pathogenetic mechanisms underlying this disease.

Acknowledgements

The authors wish to thank the patients for their cooperation.

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