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Original Scientific Paper

HLA II ALLELES AND SUSCEPTIBILITY TO MULTIPLE SCLEROSIS IN CROATIA

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SUMMARY – The aim of the present case-control study was to evaluate associations between *HLA-DRB1* and *HLA-DQB1* alleles, genotypes and haplotypes to multiple sclerosis (MS) susceptibility in the Croatian population. *HLA-DRB1* and *HLA-DQB1* genotyping was performed on a group of 173 relapsing-remitting MS patients and 205 healthy voluntary blood donors without a history of demyelization diseases. HLA class II genes were analyzed using the PCR-SSP method (*CareDx AB*, Sweden). Our results demonstrated a positive association for alleles *HLA-DRB1*15:01* (odds ratio [OR]=3.17; *P corrected* [*Pc*]=0.004), -*DQB1*06:02* (OR=2.87; *Pc*=0.002) and haplotype *HLA-DRB1*15:01~DQB1*06:02* (OR=2.75; *Pc*=0.001) that withstood correction for multiple comparisons. Risk factors identified as significantly predisposing for MS, but only prior to Bonferroni correction were: *HLA-DQB1*03:02* allele (*P*=0.016), *HLA-DRB1*01:01/*13:01* (*P*=0.033), -*DRB1*07:01/*15:01* (*P*=0.045) and -*DQB1*03:02/*06:02* genotypes (*P*=0.031) and *HLA-DRB1*04:02~DQB1*03:02* (*P*=0.034) and *HLA-DRB1*07:01~DQB1*03:03* haplotypes (*P*=0.019). A protective effect against MS was shown by alleles *HLA-DRB1*11:01* (*P*=0.004) and -*DQB1*03:01* (*P*=0.014) and haplotype *HLA-DRB1*11:01~DQB1*03:01* (*P*=0.013), but also only before the Bonferroni correction. In conclusion, our results confirm that HLA class II alleles and their interactions modulate MS susceptibility and protection in the Croatian population.

Keywords: Multiple sclerosis; HLA class II alleles; Susceptibility; Protection

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Introduction

Multiple sclerosis is a chronic inflammatory and demyelinating disorder of the central nervous system. More than 2 million young adults worldwide have MS and approximately half of the patients are residents of Europe[1]. The disease typically begins between 20 and 40 years of age and the prevalence of MS is higher in women, who are affected approximately twice as often as men[1]. Although the pathogenesis of MS is not wholly understood yet, current theories involve an association between genetic, environmental and immune factors[2,3]. It has commonly been assumed that MS is the result of an activation of autoreactive T lymphocytes and complex interactions of T and B cells in enhancing the immune response[3]. Genome-wide association studies have distinguished more than 200 risk variants associated to MS susceptibility, confirming the greatest risk is located in human leukocyte region (HLA) on chromosome 6p21.3; more precisely by the *HLA-DRB1* locus, specifically the *HLA-DR15* haplotype (*HLA-DRB1*15:01~DQA1*01:02~DQB1*06:02*), and *HLA-DRB1*15:01* was identified as the main susceptibility allele in MS[4,5]. Additional *HLA-DRB1* alleles were found to be associated with MS risk across different ethnic groups: *HLA-DRB1*13:03, HLA-DRB1*03:01, HLA-DRB1*08:01, HLA-DRB1*04:05, DRB1*15:03*[6-8]*,* but in contrast *DRB1*01, DRB1*07, HLA-DRB1*11:01, DRB1*14, DRB*13* genes/alleles were mainly considered as protective factors for MS[9,10]. Regarding the *HLA-DQB1* locus, the most widely reported association with MS was the one with the *HLA-DQB1*06:02* allele[8,10]. However, the extensive polymorphism and strong linkage disequilibrium of the *DRB1*15~DQB1*06:02* haplotype make it difficult to locate the primary predisposing allele. In African-Brazilian patients, the *HLA-DQB1*06:02* allele, and not *HLA-DRB1*15:01,* plays a primary role in MS susceptibility[11]. Conversely, no association of *HLA-DQB1* alleles was detected for African Americans[7]. Recent patient cohorts of European ancestry have reported *HLA-DQB1*03:02* as a risk factor for MS[6].

A genotype and haplotype association with disease susceptibility or resistance, rather than a single allele association, was confirmed in many studies. *HLA-DRB1*08* increases the risk of MS with *HLA-DRB1*15* in trans, while *HLA-DRB1*15* homozygotes show a dominant dose effect[12]. *HLA-DRB1*11* reduces and *HLA-DRB1*14* completely cancels the risk associated with HLA-*DRB1*15* when they are inherited together[13]. While MS is associated with the *HLA-DRB15* haplotype in most studied European populations[4,14-20], other *HLA-DRB1~DQB1* haplotypes were linked to MS propensity in the Mediterranean region: *DRB1*13:03~DQB1*03:01, DRB1*04:05~DQB1*03:01, DRB1*04~DQB1*03:02* and *DRB1*03:01~DQB1*02:01*[19-21].

Data from 2018 have reported that MS prevalence in Croatia, as a country of Southeast and Mediterranean Europe, is 143.8 per 100,000 people, which places it in the group of countries with the highest disease prevalence, like the United Kingdom and the Scandinavian countries[22]. HLA class II alleles have been well characterized in the Croatian population. The results of those studies have indicated that one of the main peculiarities of the Croatian population is great heterogeneity, which could be the result of an admixture with neighboring and immigrated populations during history (Hungarians, Austrians, Germans, Turks)[23,24]. Crnic-Martinovic *et al.* have previously analyzed the HLA class II gene polymorphism using two-digit resolution genotyping in 42 patients with MS and 63 healthy controls from the region of Gorski kotar (central Croatia), a high-risk zone for multiple sclerosis[25].

The aim of this case-control study was to analyze, for the first time, HLA II genes using "high resolution" (4-digit) genotyping on a larger number of participants to assess the risk related to *HLA-DRB1* and *HLA-DQB1* loci and multiple sclerosis in Croatian patients.

Materials and methods

Subjects

We analyzed 173 patients with the relapsing-remitting subtype of MS diagnosed by the 2010 McDonald criteria[26]. The patients' diagnoses were confirmed at the neurology center of University Hospital "Sveti Duh" by a neurologist who is an expert for MS. The control group included 205 healthy controls without a history of autoimmune diseases or neurological disorders. These subjects were recruited from voluntary blood donors at the Croatian Institute of Transfusion Medicine (CITM), Zagreb. The studied population (patients and controls) was mostly comprised of residents of the Northwestern part of Croatia. Ethical approval was given by the Ethics Committees of University Hospital "Sveti Duh", the Croatian Institute of Transfusion Medicine and School of Medicine, University of Zagreb. All participants provided written informed consent. The following demographic and clinical data were recorded: sex, age at enrolment for controls and patients, and age at disease onset for patients.

HLA genotyping

DNA was extracted from cells of the buffy coat using a commercial QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany). High-resolution typing of *HLA-DRB1* and *-DQB1* alleles was performed by the PCR-SSP method using commercial kits (CareDx AB, Sweden). Results were interpreted with the provided worksheets or with HELMBERG SCORE Software (W.M.C. Helmberg, Austria).

Statistical analysis

Allele frequencies in MS patients and controls were calculated by direct counting. Median, standard deviation (SD), mean and interquartile range for demographic characteristics of enrolled participants were calculated. Comparison between patients with MS and controls for categorical variables of sex was performed with a Chi-square test and for mean ages across the groups with the Mann-Whitney test. Comparisons of allele, genotype and haplotype frequencies between cases and controls were carried out by Chi-square of Fisher's test. The odds ratio (OR) with 95% confidence interval (CI) was estimated. All *P*-values < 0.05 were considered significant. To avoid potential artefacts due to a small number of samples, we analyzed only those alleles carried by at least five individuals in either patients or controls. The Bonferroni correction for multiple testing was applied multiplying the obtained *P*-values by the number of comparisons considered at each locus (*P corrected* – *Pc*). Haplotype frequencies were calculated using the Arlequin 3.11 software[27].

Results

Participant characteristics

Demographic data of the study population are summarized in Table 1. Among 173 patients with MS, there were 132 (76.3%) females and 41 (23.7%) males. The mean age at the onset of symptoms was 36.3 ± 11.0 years. The control group consisted of 149 males (72.7%) and 56 females (27.3%), with a mean age of 40.0 ± 11.4 years. Although there was a statistical significance in sex distribution between MS patients and controls in our study, we used data only for comparing the difference between allele, genotype and haplotype frequencies in cases and controls.

Association of HLA-DRB1 alleles with MS

The frequencies of *HLA-DRB1* alleles in patients with MS and controls are shown in Table 2. *HLA-DRB1*15:01* was significantly more present among MS patients in comparison to controls, even after the Bonferroni correction for 40 alleles ($P < 0.0001$, $Pc = 0.004$, OR = 3.17). The *HLA-DRB1*11:01* allele was found to be less frequent in MS patients than in the control group, but the statistical significance was lost after the Bonferroni correction ($P = 0.004$, $Pc = 0.160$, OR = 0.39) (Table 2).

Table 1. Demographic characteristics of MS patients and controls

	MS Patients (n=173)	**Controls (n=205)**	***P-value***
Sex, female, n (%)	132 (76.3)	56 (27.3)	< 0.001
Sex, male, n (%)	41 (23.7)	149 (72.7)	< 0.001
Age, mean, SD (IQR)	43.0 ± 11.6 (34-51)	40.0 ± 11.4 (32-49)	0.024
Age onset of symptoms, mean, SD (IQR)	36.3 ± 11.0 (27-44)		

IQR = interquartile range; MS = multiple sclerosis; SD = standard deviation.

Table 2. Distribution of HLA-DRB1 allele frequencies among patients with multiple sclerosis (n = 173) and the control group (n = 205)

HLA-DRB1* alleles	**MS patients, n (%)**	**Controls, n (%)**	**OR (95% CI)**	***P*-value**
01:01	30 (8.7)	39 (9.5)		NS
01:02	3 (0.9)	5 (1.4)		NS
03:01	29 (8.4)	42(10.2)		NS
04:01	11 (3.2)	13 (3.2)		NS
04:02	12 (3.5)	7 (1.7)		NS
04:04	5 (1.4)	8 (1.9)		NS
07:01	25 (7.2)	27 (6.6)		NS
08:01	10 (2.9)	14 (4.0)		NS
08:04	1 (0.3)	7 (1.7)		NS
10:01	5 (1.4)	6 (1.5)		NS
11:01	**16 (4.6)**	**40 (9.8)**	**0.39 (0.21-0.74)**	**0.004**
11:03	6 (1.7)	3 (0.7)		NS
11:04	21 (6.1)	30 (7.3)		NS
12:01	4 (1.2)	9 (2.2)		NS
13:01	28 (8.1)	27 (6.6)		NS
13:02	10 (2.7)	16 (3.9)		NS
14:54	4 (1.2)	9 (2.2)		NS
15:01	**69 (19.9)**	**35 (8.5)**	**3.17 (1.95-5.17)**	**< 0.0001[a]**
15:02	7 (2.0)	4 (1.0)		NS
16:01	22 (6.4)	43 (10.5)		NS

CI = confidence interval; MS = multiple sclerosis; NS = not significant; OR = odds ratio. [a] *P*-value remains significant after Bonferroni correction for 40 tested *HLA-DQB1* alleles. Alleles significantly associated with MS are shown in bold.

Table 3. Distribution of HLA-DQB1 allele frequencies among patients with multiple sclerosis (n = 173) and the control group (n = 205)

***HLA-DQB1** alleles**	**MS patients, n (%)**	**Controls, n (%)**	**OR (95% CI)**	***P-value***
02:01	31 (9.0)	44 (10.7)		NS
02:02	14 (4.0)	23 (5.6)		NS
03:01	**57 (16.5)**	**97 (23.7)**	**0.59 (0.38-0.90)**	**0.014**
03:02	**33 (9.5)**	**22 (5.4)**	**2.07 (1.15-3.72)**	**0.016**
03:03	12 (3.5)	5 (1.2)		NS
04:02	7 (2.0)	15 (3.7)		NS
05:01	38 (11.0)	54 (13.2)		NS
05:02	29 (8.4)	48 (11.7)		NS
05:03	9 (2.6)	14 (3.4)		NS
06:01	6 (1.7)	4 (1.0)		NS
06:02	**66 (19.1)**	**34 (8.3)**	**2.87 (1.76-4.69)**	**< 0.0001[a]**
06:03	30 (8.7)	28 (6.8)		NS
06:04	10 (2.9)	10 (2.4)		NS

CI = confidence interval; MS = multiple sclerosis; NS = not significant; OR = odds ratio. [a]*P*-value remains significant after Bonferroni correction for 18 tested *HLA-DQB1* alleles. Alleles significantly associated with MS are shown in bold.

Table 4. Distribution of HLA-DRB1 and HLA-DQB1 genotype frequencies in patients with multiple sclerosis (n = 173) and controls (n = 205)

HLA genotypes		**MS patients, n (%)**	**Controls, n (%)**	**OR (95% CI)**	***P-value***
DRB1 genotypes	***01:01/*13:01**	**10 (5.8)**	**3 (1.5)**	**4.13 (1.12-15.26)**	**0.033**
	**03:01/*15:01*	9 (5.2)	3 (1.5)		NS
	**03:01/*13:01*	1 (0.6)	8 (3.9)		NS
	***07/01/*15:01**	**7 (4.0)**	**1 (0.5)**	**8.60 (1.05-70.62)**	**0.045**
	**07:01/*16:01*	0	6 (2.9)		NS
	**11:01/*15:01*	4 (2.3)	5 (2.4)		NS
	**11:01/*16:01*	3 (1.7)	5 (2.4)		NS
DQB1 genotypes	**02:01/*03:01*	4 (2.3)	14 (6.8)		NS
	**02:01/*03:02*	6 (3.5)	0 (0)		NS
	**02:01/*06:02*	9 (5.2)	3 (1.5)		NS
	**02:01/*06:03*	1 (0.6)	7 (3.4)		NS
	**02:02/*03:01*	1 (0.6)	8 (3.9)		NS
	**03:01/*03:01*	5 (2.9)	10 (4.9)		NS
	**03:01/*03:02*	2 (1.2)	8 (3.9)		NS
	**03:01/*05:01*	8 (4.6)	7 (3.4)		NS
	**03:01/*05:02*	8 (4.6)	10 (4.9)		NS
	**03:01/*06:02*	10 (5.8)	12 (5.9)		NS
	***03:02/*06:02**	**8 (4.6)**	**1 (0.5)**	**9.89 (1.22-79.89)**	**0.031**
	**05:01/*06:03*	10 (5.8)	5 (2.4)		NS
	**05:02/*05:02*	5 (2.9)	6 (2.9)		NS

CI = confidence interval; MS = multiple sclerosis; NS = not significant; OR = odds ratio. Genotypes significantly associated with MS are shown in bold. Only *HLA-DRB1* and *HLA-DQB1* genotypes carried by at least five individuals in either cases or controls were analyzed.

Association of HLA-DQB1 alleles with MS susceptibility

The frequencies of *HLA-DQB1* alleles in patients with MS and controls are shown in Table 3. For the *HLA-DQB1* locus, 18 different alleles were detected. *HLA-DQB1*06:02* and *HLA-DQB1*03:02* were significantly associated with MS, but only HLA-*DQB1*06:02* remained significant after the Bonferroni correction for 18 alleles ($P<0.0001$, $Pc=0.002$, OR = 2.87). *HLA-DQB1*03:01* ($P=0.014$, $Pc=0.252$, OR = 0.59) was found to be underrepresented in MS patients, but did not remain significant after the Bonferroni correction.

Association of HLA-DRB1 and -DQB1 genotypes with MS

Among *HLA-DRB1* genotypes, *HLA-DRB1*01:01/*13:01* ($P=0.033$) and *HLA-DRB1*07:01/*15:01* ($P=0.045$) were found to be positively associated with MS among Croatian patients (Table 4). For *HLA-DQB1* genotypes, an increased frequency of *DQB1*03:02/*06:02* ($P=0.031$) was found in MS patients. All those associations lost significance after the Bonferroni correction for the 9 *HLA-DRB1* and 17 *HLA-DQB1* genotypes that were included.

Association of HLA-DRB1~DQB1 haplotypes with MS

The association of *HLA-DRB1~DQB1* haplotypes with MS risk was examined and only haplotypes documented in at least 1% of the sample cases of controls were considered (Table 5). Three haplotypes were found to be significantly positively associated: *HLA-DRB1*04:02~DQB1*03:02* (OR = 4.13, *P* = 0.034), *HLA-DRB1*07:01~DQB1*03:03* (OR = 6.23, *P* = 0.019) and *HLA-DQB1*15:01~DRB1*06:02* (OR = 2.75, *P* < 0.0001). The *HLA-DRB1*11:01~DQB1*03:01* (OR = 0.42, *P* = 0.013) haplotype was found to be negatively associated with MS. Only *HLA-DRB1*15:01~DQB1*06:02* remained significant after the Bonferroni correction for 15 haplotypes included in our analysis (*Pc* = 0.001).

Discussion

Croatia is located in Southeast and Mediterranean Europe, and has a quite high prevalence of multiple sclerosis (143.8/100,000 inhabitants)[22]. Based on our knowledge, this is the first study which analyzed *HLA-DRB1* and *HLA-DQB1* alleles at the 4-digit level and demonstrated an association between HLA class II alleles, genotypes and haplotypes and MS susceptibility in the Croatian population.

In our study, among alleles at the *HLA-DRB1* locus, the most frequent allele in the control group was *HLA-DRB1*16:01* (10.5%), followed by *HLA-DRB1*03:01* (10.2%), *-DRB1*11:01* (9.8%), *-DRB1*01:01* (9.5%), *-DRB1*15:01* (8.5%) and *-DRB1*11:04* (7.3%). The most frequent allele at the *HLA-DQB1* locus in controls was *HLA-DQB1*03:01* (23.7%), followed by *HLA-DQB1*05:01* (13.2%), *-DQB1*05:02* (11.7%), *-DQB1*02:01* (10.7%) and *-DQB1*06:02* (8.3%) The results showed that the distribution of these HLA class II alleles in our controls was similar to the results of other studies conducted in Croatia[23,24].

At the *HLA-DRB1* locus, we found a significant association between *HLA-DRB1*15:01* and susceptibility to MS in Croatian patients (*Pc* = 0.004). A previous study in Croatia also reported a significant association between the *HLA-DRB1*15* allele and MS[25]. The involvement of *HLA-DRB*15:01* in MS

Table 5. Frequencies of HLA-DRB1~DQB1 haplotypes in patients with multiple sclerosis (n = 173) and controls (n = 205)

HLA-DRB1~DQB1 haplotypes	MS patients, n (%)	Controls, n (%)	OR (95% CI)	*P-value*
**01:01~*05:01*	20 (5.8)	25 (6.1)		NS
**03:01~*02:01*	28 (8.1)	40 (9.8)		NS
**04:01~*03:02*	8 (2.3)	3 (0.7)		NS
****04:02~*03:02***	**10 (2.9)**	**3 (0.7)**	**4.13 (1.12-15.26)**	**0.034**
**07:01~*02:02*	8 (2.3)	17 (4.1)		NS
****07:01~*03:03***	**10 (2.9)**	**2 (0.5)**	**6.23 (1.35-28.82)**	**0.019**
**08:01~*04:02*	5 (1.4)	7 (1.7)		NS
**10:01~*05:01*	3 (0.9)	6 (1.5)		NS
****11:01~*03:01***	**13 (3.8)**	**33 (8.0)**	**0.42 (0.22-0.83)**	**0.013**
**11:04~*03:01*	16 (4.6)	21 (5.1)		NS
**13:01~*06:03*	24 (6.9)	18 (4.4)		NS
**13:02~*06:04*	6 (1.7)	6 (1.5)		NS
**15:01~*05:02*	7 (2.0)	3 (0.7)		NS
****15:01~*06:02***	**54 (15.6)**	**29 (7.1)**	**2.75 (1.66-4.58)**	**< 0.0001[a]**
**16:01~*05:02*	18 (5.2)	32 (7.8)		NS

CI = confidence interval; MS = multiple sclerosis; NS = not significant; OR = odds ratio. [a] *P*-value remains significant after Bonferroni correction for 15 considered haplotypes. Haplotypes significantly associated with MS are shown in bold.

propensity has been reported in neighboring countries[28-30]. *HLA-DRB1*15:01* has been identified as a primary risk factor for MS among other European populations, as demonstrated in recently published studies form Greece, Slovakia and Malta as well[16,17,31]. Interestingly, the authors from Malta pointed out that populations from Southern Europe (Sicily, Sardinia, Greece and Bulgaria) have a higher prevalence of MS (120, 157, 39, 45 per 10^{-5}, respectively) than would be expected based on their *DRB1*15* frequencies (4.3%, 4.6%, 6.8%, 5.4%, respectively) when compared to other European countries[31]. The same observation can be related to our study, because we found a frequency of *HLA-DRB1*15:01* (8.5%) similar to Southern Europe, but the prevalence of MS (143.8/100,000)[22] was comparable with countries of Northern Europe, such as Scandinavian countries and the United Kingdom (150/100,000 or greater)[1]. The strength of the *DRB1*15:01* association with MS in our study is similar to the strength of this association found in most other studies conducted in European populations[2] and increases the risk of MS three-fold (OR = 3.17).

For the *HLA-DRB1* locus, we identified *HLA-DRB1*11:01* as a protective allele (*P* = 0.004; OR = 0.39), although this association did not withstand the correction for the number of comparisons. The frequency of *HLA-DRB1*11:01* was significantly lower in MS patients than in the control group, which is in line with previous findings in Italy and Malta[29,31]. The protective effect of *DRB1*11* was seen in the meta-analyses of Zhang *et al.* in Caucasians and the same has been established in Canadian and Western Australian populations[9,13,32]. Wu and coworkers have reported that the *HLA-DRB1*11:01* allele can only be determined as a protective factor when using 4-digit HLA genotyping as opposed to 2-digit typing, which is consistent with our results[32]. These authors have also pointed out that high-resolution typing allows for a more reliable recognition of both positive and negative risk associations with alleles, which may be ambiguous with 2-digit typing[32]. The association with *HLA-DRB1*04* demonstrated in a previous 2-digit genotyping study in Croatia, conducted in a high prevalence area for MS – Gorski Kotar, was not confirmed in our study, which is presumably also linked to resolution differences in HLA genotyping[25].

Our analysis of the *HLA-DQB1* alleles in relapsing-remitting MS patients and healthy controls showed a positive association with MS for *HLA-DQB1*06:02* and *-DQB1*03:02* alleles and a negative association for the *HLA-DQB1*03:01* allele. *HLA-DQB1*06:02* was significantly associated with risk for MS, even after the Bonferroni correction for 18 alleles (OR = 2.87, *Pc* = 0.002). Similar findings have been reported by Crnic-Martinovic and coworkers in a previous MS study in Croatia[25]. Fernandez *et al.* have shown that the *HLA-DQB1*06:02* allele was the only MS risk factor which maintained association by logistic regression analysis in Malaga, Spain[14]. A positive association with MS and the *HLA-DQB1*06:02* allele was obtained by studies from Greece, Slovakia and Belgium[16,17,18]. A study of Afro-Brazilian MS patients has demonstrated that the *HLA-DQB1*06:02* allele increases the risk for MS in the absence of the *HLA-DRB1*15:01* allele[11]. In contrast, the studies carried out in African Americans and patients from Sicily have showed a primary association between MS and the *HLA-DRB1*15:01* allele without any association with the *HLA-DQB1*06:02* allele[7,20]. A study on "humanized" mice transgenic for HLA has shown that susceptibility to MS-like disease induced by the myelin oligodendrocytic basic protein or by the proteolipid protein is determined by *HLA-DQB1*06:02* rather than *HLA-DRB1*15:01*[33]. We observed a higher frequency of *HLA-DQB1*03:01* in controls than in MS patients (23.7% vs 16.5%; *P* = 0.014), whereas *HLA-DQB1*03:02* was found more frequently in MS patients (9.5% vs 5.4%, *P* = 0.016). All these *HLA-DQB1*03* associations, however, lost significance after the Bonferroni correction for 18 alleles. There have been reports of an association of *HLA-DQB1*03:01* with MS risk. Werneck *et al.* and Kolaee *et al.* have found a lower prevalence of *HLA-DQB1*03:01* in patients with MS compared to controls (8.8% vs. 19.1% and 20% vs. 29.2% respectively)[34,35]. However, *HLA-DQB1*03:01* was identified as a predisposing allele in MS patients from the Spanish province of Biscay, but the significance was lost after the Bonferroni correction[15]. The results of the analyses of SNP data from 11 MS patient cohorts of European ancestry revealed *HLA-DQB1*03:02* as a dominant risk allele, but that the risk effect can be cancelled by an allelic interaction between *HLA-DQB1*03:01* and *HLA-DQB1*03:02* alleles[6]. A study conducted in the Slovak population has demonstrated an association between the *HLA-DQB1*03* gene and a higher susceptibility for MS[17]. In this study, we did not identify *HLA-DQB1*03:03* as a high-risk allele for the development of MS as reported in a previous study performed in the region of Gorski kotar, Croatia[25].

Recent studies have indicated the importance of epistatic interactions among the *HLA-DRB1* alleles that affect the risk of MS. Considering this, we investigated how much the interaction between alleles comprising *HLA-DRB1* and *-DQB1* genotypes determine MS risk in the Croatian population. Our findings demonstrated that *HLA-DRB1*01:01/*13:01* ($P = 0.033$), *HLA-DRB1*07:01/*15:01* ($P = 0.045$) and *HLA-DQB1*03:02/*06:02* ($P = 0.031$) genotypes were found to be overrepresented in Croatian MS patients, however only before corrections for the number of comparisons. Ramagopalan *et al.* reported that the *HLA-DRB1*07/*15* genotype increased the risk of MS compared to the other *HLA-DRB1*15* heterozygotes (OR = 1.28, $P = 0.038$)[13]. A study conducted in Western Australia has shown higher frequencies of the *HLA-DRB1*07/*05* genotype in MS patients[32]. Unlike our findings, an Iranian study observed that the *DRB1*15:01/*07:01*⁻ genotype is significantly associated with MS susceptibility (OR = 3.320, $P = 0.006$)[35]. To our knowledge, the relation between *HLA-DRB1*01:01/*13:01* and a greater risk of MS has not yet been reported. Moreover, *HLA-DRB1*01* and *DRB1*13* alleles were found to be protective factors in Caucasians[9]. On the other hand, Romero-Pinel *et al.* reported that *HLA-DRB1*01* alleles are associated with a worse prognosis of MS[36].

In this study, we also analyzed the association between *HLA-DRB1~DQB1* haplotypes and MS susceptibility. We demonstrated a positive correlation between MS propensity and the *HLA-DRB1*15:01~DQB1*06:02* haplotype (OR = 2.75, $P < 0.0001$), even after correction for the 15 considered haplotypes. This result is in line with other studies performed in populations of Northern-European ancestry, Mediterranean Europeans (Greeks, Italians, Spaniards) and the Central European Slovak population[4,14-17,19,21]. A previous study from Croatia identified the *HLA-DRB1*15~DQA1*01:02~DQB1*06:02* haplotype as a risk factor significantly associated with the secondary-progressive subtype of MS[25]. A positive association in our study was found for the *HLA-DRB1*04:02~DQB1*03:02* haplotype and MS (OR = 4.13, $P = 0.034$). The same observations were shown in MS patients from the province of Biscay (Spain), Sicily and Turkey[15,20,30]. On Sardinia, MS is associated with a different subtype of the DR4 haplotype: *HLA-DRB1*04:05~DQA1*05:01~DQB1*03:01*[19,21]. These findings may be relevant as the southern part of Croatia belongs to the Mediterranean region. Association with DR4 seems to be typical for Mediterranean countries and we suppose that a significantly higher frequency of the *HLA-DRB1*04:02~DQB1*03:02* haplotype in our study is a result of migration from the Mediterranean South to the economically more developed Northwestern part of Croatia.

The only protective HLA haplotype for MS development in our study was *HLA-DRB1*11:01~DQB1*03:01* (OR = 0.42, $P = 0.013$). In the Sardinian population, among *HLA-DRB1~DQB1* haplotypes, haplotypes carrying *HLA-DRB1 *11* were found to be a protective factor[21]. The study from Slovakia conducted on 282 patients with sporadic MS also showed the *HLA-DRB1*11~DQB1*03* haplotype as a protective against MS development[17].

Our findings are consistent with those in other countries, although a great number of studies was performed using 2-digit genotyping, which makes the comparison of our results difficult. An additional limitation of our study could also be the fact that all examined associations must be confirmed on a larger number of participants, especially on genotype and haplotype level.

Conclusion

In conclusion, our results demonstrated an association of HLA class II genes on allele, genotype and haplotype level with MS susceptibility in the Croatian population using high resolution HLA typing techniques, with a special emphasis on the significant positive association with *HLA-DRB1*15:01* and *HLA-DQB1*06:02* alleles and the *HLA-DRB1*15:01~DQB1*06:02* haplotype.

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References

1. Kingwell E, Marriott JJ, Jetté N, Pringsheim T, Makhani N, Morrow SA, *et al.* Incidence and prevalence of multiple sclerosis in Europe: a systematic review. BMC Neurol. 2013;13:128. https://doi.org/10.1186/1471-2377-13-128
2. Didonna A, Oksenberg JR. Genetic determinants of risk and progression in multiple sclerosis. Clin Chim Acta. 2015;449:16–22. https://doi.org/10.1016/j.cca.2015.01.034
3. Lazibat I, Majdak MR, Županić S. Multiple sclerosis: New aspects of immunopathogenesis. Acta Clin Croat. 2018;57:352-361. https://doi.org/10.20471/acc.2018.57.02.17
4. Sawcer S, Hellenthal G, Pirinen M, Spencer CCA, Patsopoulos NA, Moutsianas L, *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476:214–219. https://doi.org/10.1038/nature10251
5. International Multiple Sclerosis Genetics Consortium. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. Science. 2019;365:eaav7188. https://doi.org/10.1126/science.aav7188
6. Moutsianas L, Jostins L, Beecham AH, Dilthey AT, Xifara DK, Ban M, *et al.* Class II HLA interactions modulate genetic risk for multiple sclerosis. Nat Genet. 2015;47:1107–1113. https://doi.org/10.1038/ng.3395
7. Isobe N, Gourraud PA, Harbo HF, Caillier SJ, Santaniello A, Khankhanian P, *et al.* Genetic risk variants in African Americans with multiple sclerosis. Neurology. 2013;81:219–227. https://doi.org/10.1212/WNL.0b013e31829bfe2f
8. Rojas OL, Rojas-Villarraga A, Cruz-Tapias P, Sánchez JL, Suárez-Escudero JC, Patarroyo MA, *et al.* HLA class II polymorphism in Latin American patients with multiple sclerosis. Autoimmun Rev. 2010;9:407-413. https://doi.org/10.1016/j.autrev.2009.11.001
9. Zhang Q, Lin CY, Dong Q, Wang J, Wang W. Relationship between HLA-DRB1 polymorphism and susceptibility or resistance to multiple sclerosis in Caucasians: a meta-analysis of non-family-based studies. Autoimmun Rev. 2011;10:474–481. https://doi.org/10.1016/j.autrev.2011.03.003
10. Lima TFR, Braga VLL, Silva JTD, Simplício GN, De Oliveira SÁA, Barros RR, *et al.* The HLA-DRB1 alleles effects on multiple sclerosis: A systematic review. Int Arch Med. 2015;8. https://doi.org/10.3823/1687
11. Caballero A, Alvés-León S, Papais-Alvarenga R, Fernández O, Navarro G, Alonso A. DQB1*0602 confers genetic susceptibility to multiple sclerosis in Afro-Brazilians. Tissue Antigens. 1999;54:524-526. https://doi.org/10.1034/j.1399-0039.1999.540511.x
12. Barcellos LF, Sawcer S, Ramsay PP, Baranzini SE, Thomson G, Briggs F, *et al.* Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. Hum Mol Genet. 2006;15:2813–2824. https://doi.org/10.1093/hmg/ddl223
13. Ramagopalan SV, Morris AP, Dyment DA, Herrera BM, DeLuca GC, Lincoln MR, *et al.* The inheritance of resistance alleles in multiple sclerosis. PLoS Genet. 2007;3:1607-1613. https://doi.org/10.1371/journal.pgen.0030150
14. Fernández O, Fernández V, Alonso A, Caballero A, Luque G, Bravo M, *et al.* DQB1*0602 allele shows a strong association with multiple sclerosis in patients in Malaga, Spain. J Neurol. 2004;251:440–444. https://doi.org/10.1007/s00415-004-0350-2
15. Fernández O, R-Antigüedad A, Pinto-Medel MJ, Mendibe MM, Acosta N, Oliver B, *et al.* HLA class II alleles in patients with multiple sclerosis in the Biscay province (Basque Country, Spain). J Neurol. 2009;256:1977-1988. https://doi.org/10.1007/s00415-009-5223-2
16. Kouri I, Papakonstantinou S, Bempes V, Vasiliadis HS, Kyritsis AP, Pelidou SH. HLA associations with multiple sclerosis in Greece. J Neurol Sci. 2011;308:28–31. https://doi.org/10.1016/j.jns.2011.06.037
17. Michalik J, Čierny D, Kantorová E, Kantárová D, Juraj J, Párnická Z, *et al.* The association of HLA-DRB1 and HLA-DQB1 alleles with genetic susceptibility to multiple sclerosis in the Slovak population. Neurol Res. 2015;37:1060-1067. https://dx.doi.org/10.1080/01616412.2015.1115212
18. Lysandropoulos AP, Racapé J, Holovska V, Toungouz M. Human leucocyte antigen (HLA) class I and II typing in Belgian multiple sclerosis patients. Acta Neurol Belg. 2017;117:61–65. https://doi.org/10.1007/s13760-016-0716-0
19. Marrosu MG, Murru R, Murru MR, Costa G, Zavattari P, Whalen M, *et al.* Dissection of the HLA association with multiple sclerosis in the founder isolated population of Sardinia. Hum Mol Genet. 2001;10:2907–2916. https://doi.org/10.1093/hmg/10.25.2907
20. Brassat D, Salemi G, Barcellos LF, McNeill G, Proia P, Hauser SL, *et al.* The HLA locus and multiple sclerosis in Sicily.

Neurology. 2005;64:361-363. https://doi.org/10.1212/01.WNL.0000149765.71212.0A

21. Cocco E, Murru R, Costa G, Kumar A, Pieroni E, Melis C, *et al.* Interaction between HLA-DRB1-DQB1 haplotypes in Sardinian multiple sclerosis population. PLoS One. 2013;8:e59790. https://doi.org/10.1371/journal.pone.0059790
22. Benjak T, Štefančić V, Draušnik Ž, Cerovečki I, Roginić D, Habek M, *et al.* Prevalence of multiple sclerosis in Croatia: data from national and non-governmental organization registries. Croat Med J. 2018;59:65-70. https://doi.org/10.3325/cmj.2018.59.65
23. Grubić Z, Žunec R, Čečuk-Jeličić E, Kehrin-Brkljačić V, Kaštelan A. Polymorphism of HLA-A, -B, -DRB1, -DQA1 and -DQB1 haplotypes in a Croatian population. Eur J Immunogenet. 2000;27:47-51 https://doi.org/10.1046/j.1365-2370.2000.00193.x
24. Grubic Z, Burek Kamenaric M, Mikulic M, Stingl Jankovic K, Maskalan M, Zunec R. HLA-A, HLA-B and HLA-DRB1 allele and haplotype diversity among volunteer bone marrow donors from Croatia. Int J Immunogenet. 2014;41:211-221. https://doi.org/10.1111/iji.12117
25. Crnic-Martinovic M, Balen S, Katalinic N, Fucak M, Grahovac B, Sepcic J, *et al.* HLA Class II gene polymorphism in multiple sclerosis patients from Gorski Kotar, Croatia. Tissue Antigens. 2010;75:622-623 (P321).
26. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011;69:292–302. https://doi.org/10.1002/ana.22366
27. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online. 2007;1:47–50. https://doi.org/10.1177/117693430500100003
28. Schmied MC, Zehetmayer S, Reindl M, Ehling R, Bajer-Kornek B, Leutmezer F, et al. Replication study of multiple sclerosis (MS) susceptibility alleles and correlation of DNA-variants with disease features in a cohort of Austrian MS patients. Neurogenetics. 2012;13:181–187. https://doi.org/10.1007/s10048-012-0316-y
29. Ballerini C, Guerini FR, Rombolà G, Rosati E, Massacesi L, Ferrante P, et al. HLA–multiple sclerosis association in Continental Italy and correlation with disease prevalence in Europe J Neuroimmunol. 2004;150:178-185. https://doi.org/10.1016/j.jneuroim.2004.01.015
30. Saruhan-Direskeneli G, Esin S, Baykan-Kurt B, Ornek I, Vaughan R, Eraksoy M. HLA-DR and -DQ Associations with multiple sclerosis in Turkey. Hum Immunol. 1997;55:59-65. https://doi.org/10.1016/S0198-8859(97)00086-4
31. Dean G, Yeo TW, Goris A, Taylor CJ, Goodman RS, Elian M, *et al.* HLA-DRB1 and multiple sclerosis in Malta. Neurology. 2008;70:101-105. https://doi.org/10.1212/01.wnl.0000284598.98525.d7
32. Wu J-S, James I, Wei Qiu, Castley A, Christiansen FT, Carroll WM, et al. Influence of HLA-DRB1 allele heterogeneity on disease risk and clinical course in a West Australian MS cohort: a high-resolution genotyping study. Mult Scler. 2010;16:526–532. https://doi.org/10.1177/1352458510362997
33. Kaushansky N, Ben-Nun A. DQB1*06:02-Associated Pathogenic Anti-Myelin Autoimmunity in Multiple Sclerosis-Like Disease: Potential Function of DQB1*06:02 as a Disease-Predisposing Allele. Front Oncol. 2014;4:280. https://doi.org/10.3389/fonc.2014.00280
34. Werneck LC, Lorenzoni PJ, Arndt RC, Kay CSK, Scola RH. The immunogenetics of multiple sclerosis. The frequency of HLA-alleles class 1 and 2 is lower in Southern Brazil than in the European population. Arq Neuropsiquiatr. 2016;74:607-616. https://doi.org/10.1590/0004-282X20160100
35. Kollaee A, Ghaffarpor M, Ghlichnia HA, Ghaffari SH, Zamani M. The influence of the HLA-DRB1 and HLA-DQB1 allele heterogeneity on disease risk and severity in Iranian patients with multiple sclerosis. Int J Immunogenet. 2012;39:414–422. https://doi.org/10.1111/j.1744-313X.2012.01104.x
36. Romero-Pinel L, Pujal JM, Martínez-Yélamos S, Gubieras L, Matas E, Bau L, et al. HLA-DRB1: genetic susceptibility and disability progression in a Spanish multiple sclerosis population. Eur J Neurol. 2011;18:337–342. https://doi.org/10.1111/j.1468-1331.2010.03148.x

Sažetak

HLA II ALELI I PODLOŽNOST MULTIPLOJ SKLEROZI U HRVATSKOJ

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Cilj rada bio je istražiti povezanost *HLA-DRB1* i *HLA-DQB1* alela, genotipova te haplotipova s podložnošću za nastanak multiple skleroze (MS) u hrvatskoj populaciji metodom istraživanja parova. *HLA-DRB1* i *HLA-DQB1* genotipizacija učinjena je kod 173 pacijenta s relapsno-remitirajućim oblikom MS i 205 zdravih dobrovoljnih darivatelja krvi bez povijesti demijelinizacijskih bolesti kao kontrolnom skupinom. Geni HLA razreda II određeni su metodom PCR-SSP korištenjem komercijalnih testova (CareDx AB, Švedska). Aleli *HLA-DRB1*15:01* (omjer izgleda [OR] = 3,17; *P* uz Bonferronijevu korekciju [*Pc*] = 0,004), *-DQB1*06:02* (OR = 2,87; *Pc* = 0,002) i haplotip *HLA-DRB1*15:01~DQB1*06:02* (OR = 2,75; *Pc* = 0,001) statistički su učestaliji u oboljelih od MS u odnosu na kontrolnu skupinu. Rizični čimbenici povezani s nastankom MS, ali prije korekcije *P* vrijednosti, bili su: alel *HLA-DQB1*03:02* (*P* = 0,016), genotipovi *HLA-DRB1*01:01/*13:01* (*P* = 0,033), *DRB1*07:01/*15:01* (*P* = 0,045) i *DQB1*03:02/*06:02* (*P* = 0,031) te haplotipovi *HLA-DRB1*04:02~DQB1*03:02* (*P* = 0,034) i HLA-*DRB1*07:01~DQB1*03:03* (*P* = 0,019). Među MS bolesnicima smanjena je zastupljenost alela *HLA-DRB1*11:01* (*P* = 0,004) i *DQB1*03:01* (*P* = 0,014) te haplotipa HLA-*DRB1*11:01~DQB1*03:01* (*P* = 0,013), ali samo prije Bonferronijeve korekcije. U zaključku, naši rezultati ukazuju na to da navedeni aleli HLA razreda II utječu na podložnost ili pokazuju zaštitnu ulogu u nastanku MS u hrvatskoj populaciji.

Ključne riječi: *Multipla skleroza; HLA aleli razreda II; Podložnost; Zaštita*